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REMARKS

Claims 1, 3-7 and 13-16 are pending and were subject to examination in the outstanding Office Action. Claims 1 and 16 are amended above to put the application in better condition for allowance or appeal. Applicants note with appreciation that Claims 4-6 and 14 are allowed and Claims 1, 3, 7 and 13 are only subject to objections. The remaining issues raised in the Office Action are addressed below.

I.   Oath/Declaration.

The Office Action states that the Declaration is defective. Applicants submitted a new Declaration executed by the inventors on October 30, 2003. Accordingly, Applicants respectfully request that this objection be withdrawn.

II.   Priority Claim.

The Office Action states that the priority claim to U.S. Provisional Application No. 60/166,927 is acknowledged, but refers to errors in the original Declaration in listing the priority application. As noted above, a new Declaration has been submitted claiming priority to both U.S. Provisional Application Nos. 60/166,927 and 60/127,261.

III.   Claim Objections.

Claims 1, 3, 7 and 13 remain objected to because of the recitation of "ROC1." Although Applicants disagree with this objection, Applicants have amended Claim 1 to recite "Regulator of Cullins 1 (ROC1)."

IV.   Rejection under 35 U.S.C. § 112, second paragraph.

Claim 15 stands rejected on the basis that it recites an expression vector "encoding" an antisense oligonucleotide rather than "comprising" the antisense oligonucleotide. The Office Action states: "[a]s known in the art, nucleic acids

encode polypeptides, therefore it is unclear as to how a vector can encode an oligonucleotide."

Applicants respectfully disagree with the Examiner on this point. In order to produce an antisense nucleic acid (other than by synthetic chemistry methods), the antisense oligonucleotide is generally encoded by a genetic construct such as an expression vector. Contrary to the assertion in the Office Action, it is well-known in the art that not all nucleic acids or RNAs encode polypeptides. A few examples of non-translated nucleic acids include antisense nucleic acids, RNAi, "guide" nucleic acids, snRNA, ribosomal RNA, etc.

Applicants are enclosing herewith two abstracts that explicitly describe expression vectors "encoding" antisense oligonucleotides (Mautino et al., "Inhibition of HIV-1 replication by novel lentiviral vectors expressing transdominant Rev and HIV-1 env antisense," Gene Ther. 9:421-31 (2002); Weiss et al., "Antisense strategies in neurobiology," Neurochemistry International 31:321-48 (1997)).

In view of the foregoing, the scope of Claim 15 is clear and definite to those of ordinary skill in the art. Accordingly, Applicants request withdrawal of the outstanding rejection under §112, second paragraph.

V.   Written Description.

The Office Action has maintained the rejection of Claims 15 and 16 for lack of written description. For the purposes of this rejection, Claim 15 has been construed as claiming an expression vector "comprising" an antisense oligonucleotide. As discussed in the previous section, this construction is erroneous. Claim 15 is directed to an expression vector "encoding" the antisense oligonucleotide of Claim 13.

Claim 15 is directed to an expression vector encoding the antisense oligonucleotide of Claim 13, which is 12 to 50 nucleotides in length. The written description rejections against Claims 1 and 13 have been withdrawn. Claim 15 depends from Claim 13. As Claim 13 is deemed to satisfy the written description

requirement, the subject matter of Claim 15 does as well. Claim 15 recites an expression vector encoding the antisense oligonucleotide of Claim 13. The application provides written support for the antisense oligonucleotide of Claim 13. The application further provides written support for an expression vector (page 24, lines 13-19 and page 25, lines 11-25). The application further provides written support for an expression vector encoding an antisense oligonucleotide of Claim 13 as one of ordinary skill in the art would immediately be able to envision an expression vector (e.g., a plasmid) that encodes and expresses the antisense oligonucleotide of Claim 13.

With respect to Claim 16, this claim is directed to a method of making a protein comprising the amino acid sequence of SEQ ID NO:2 or a fragment thereof by expressing the protein or fragment from an expression vector comprising a nucleic acid that (i) consists of the nucleic acid sequence of SEQ ID NO:1, (ii) a nucleic acid that has a nucleotide sequence that differs from the sequence of SEQ ID NO: due to the degeneracy of the genetic code, or (iii) a fragment of at least 60 consecutive nucleotides of (i) or (ii).

The Office Action states that:

Claim 16 is directed to a method of producing any protein of any function which comprises at least 20 amino acids of the polypeptide of SEQ ID NO:2. ... As indicated in previous Office Action Paper No. 19, many functionally unrelated polynucleotides are encompassed by the claims . . .. In addition, many structurally unrelated polynucleotides are encompassed by the claims.

Applicants respectfully disagree with this rejection. Claim 16, subparagraph (i) recites an expression vector comprising a polynucleotide comprising a nucleic acid consisting of the nucleotide sequence of SEQ ID NO:1. Clearly, a nucleic acid having the nucleotide sequence of SEQ ID NO:1 has written support in the specification. Further, contrary to the assertion in the Office Action, subparagraph (i) recites functionally- and structurally-related polynucleotides.

Likewise, in subparagraph (ii), Claim 16 recites an expression vector comprising a polynucleotide comprising a nucleic acid having a nucleotide sequence

that differs from the nucleotide sequence of SEQ ID NO:1 due to the degeneracy of the genetic code. The specification provides written support for degenerate sequences of SEQ ID NO:1 (which encode SEQ ID NO:2), and Applicants are clearly in possession of such degenerate sequences. The degenerate sequences of SEQ ID NO:1, which encode SEQ ID NO:2, recite by Claim 16, subparagraph (ii), also define a class of structurally- and functionally-related molecules.

Claim 16, subparagraph (iii) recites an expression vector comprising a polynucleotide comprising a segment of at least 60 nucleotides of the nucleic acids of subparagraph (i) (SEQ ID NO:1) or subparagraph (ii) (degenerate sequences of SEQ ID NO:1). Thus, subparagraph (iii) recites a genus of fragments of SEQ ID NO:1, or degenerate sequences thereof, that encode a fragment of at least 20 amino acids of SEQ ID NO:2. Clearly, Applicants are in possession of this genus of structurally- and functionally-related molecules. Thus, the specification provides written support for Claim 16, subparagraph (iii).

The fragments of at least 20 amino acids encoded by the nucleic acids recited by Claim 16, subparagraph (iii), can be used to produce antibodies against ROC1. Thus, Claim 16, subparagraph (iii) recites structural features sufficient to identify the recited fragments, which can be sued to produce antibodies against ROC1, *i.e.*, a segment of at least 60 nucleotides of SEQ ID NO:1 or a degenerate sequence thereof, which encodes at least 20 amino acids of the ROC1 protein.

In summary, Applicants submit that Claim 16 is drawn to a genus of structurally- and functionally-related nucleic acids and complies with the written description requirement of § 112, first paragraph.

For the foregoing reasons, Applicants request that the rejection under § 112, first paragraph against Claims 15 and 16 be withdrawn.

VI.   Enablement.

The rejection of Claims 15 and 16 for lack of enablement has been maintained.

For the purposes of this rejection, Claim 15 has been construed as directed to an expression vector "comprising" the antisense oligonucleotide of Claim 13. As addressed before, in fact, Claim 15 is directed to a nucleic acid "encoding" the antisense oligonucleotide of Claim 13. The outstanding enablement rejection has been withdrawn with respect to Claim 13. One skilled in the art would be able to make and use an expression vector (*e.g.*, a plasmid or viral vector) encoding the antisense oligonucleotide of Claim 13 without undue burden. Thus, the subject matter of Claim 15 is enabled.

Claim 16 is directed to a method of producing a protein comprising the amino acid sequence of SEQ ID NO:2 or a fragment thereof. As discussed above, Claim 16 recites a genus of structurally- and functionally-related molecules. Thus, one skilled in the art would be able practice the method of Claim 16 based on the guidance in the specification and the general knowledge in the art using no more than routine skill.

Accordingly, Applicants respectfully request that the rejection for lack of enablement under 35 U.S.C. §112, first paragraph, be withdrawn.

VII.   Rejection under 35 U.S.C. § 103.

Claim 15 stands rejected under §103 for obviousness over Arino et al. (GenBank accession number CAA99155). This rejection is respectfully traversed below.

Claim 15 depends from Claim 13 and, ultimately, from Claim 1. As addressed in the previous Amendment dated August 6, 2003, Claim 1 excludes the nucleic acids of Arino et al. Claims 1 and 13 are free of the outstanding obviousness rejection. Further, Claim 13 has been amended to recite an "antisense oligonucleotide that is 12 to 50 nucleotides in length and is completely complementary to a portion of the nucleic acid encoding ROC1 of Claim 1." Claim 15 is drawn to an expression vector encoding the antisense oligonucleotide of Claim 13. As Claims 1 and 13 are patentable over the Arino reference, Claim 15 (which

depends from Claims 1 and 13) is also patentable over Arino et al.

Arino et al. does not disclose or suggest the isolated polynucleotide of Claim 1, the antisense oligonucleotide or Claim 13, or the expression vector of Claim 15 that encodes the antisense oligonucleotide of Claim 13. Specifically, Arino et al. does not disclose an expression vector encoding an antisense oligonucleotide that is 12 to 50 nucleotides in length and completely complementary to a portion of a nucleic acid encoding ROC1. For this reason, Applicants submit that the subject matter of Claim 15 is nonobvious over Arino et al., and request that the outstanding obviousness rejection be withdrawn.

VIII. Conclusions.

The concerns of the Examiner having been addressed in full, Applicants respectfully request withdrawal of all outstanding rejections and the issuance of a Notice of Allowance forthwith. The Examiner is encouraged to address any questions regarding the foregoing to the undersigned attorney, who may be reached at (919) 854-1400.

Respectfully submitted,

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Enclosures: Mautino et al.
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Sloan Smith

Designing antisense to inhibit the renin-angiotensin system

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Abstract

Overactive renin-angiotensin system has been indicated in numerous pathological situations. Current treatment is based on pharmaceutical compounds, which work on the proteins level. Undisputedly helpful, it is not, however, flawless. Some of the drawbacks include adverse effects and non-compliance problem, since in many cases medicine has to be taken at least once a day for a long time. Therefore it seems logical to try a different approach, for instance to correct the disease at the gene expression level, possibly having a choice of shorter or longer-lasting effects. This current review combines results, relevant to the angiotensin system, with the antisense approach, which decreases amount of target protein by interfering at the mRNA level. Dependent on the tool used — oligodeoxynucleotide, plasmid or viral vector, the antisense effect lasts from few days to months. (Mol Cell Biochem **212**: 145–153, 2000)

Key words: angiotensin, antisense, oligodeoxynucleotide, plasmid, adeno-associated virus, hypertension

Introduction

Antisense nucleic acid is a sequence complementary to a sense, coding sequence.

Antisense inhibition has been developed, starting from cell culture applications through studies on animals, and reaching the point where it is being tested in clinical trials for HIV and cancer [1, 2]. The advantages and drawbacks of its use have been reviewed elsewhere [3]. Before 1992, however, antisense (AS) had not been applied *in vivo* with any success. There was much concern about the efficiency of cellular uptake of oligodeoxynucleotides (ODNs). In 1992–1993, few laboratories simultaneously and independently designed and tested AS-ODNs in the brain [4–8]. Cellular uptake was not a limiting factor in the central nervous system.

AS-ODNs have many potential attractive features as a new class of therapeutic agents to inhibit the renin-angiotensin system (RAS) [8]. Role of the components of RAS in a disease state has been described in many previous publications [9–14]. The first demonstration of antisense for reducing hypertension was the use of antisense to angiotensinogen mRNA and angiotensin II type 1 receptor (AT_1-R) mRNA in the brain [15]. To prolong the effect of antisense inhibition for weeks or months, DNA (partial or full-length) can be inserted in the antisense orientation in viral vectors.

Antisense oligonucleotides

Ideal antisense molecules

Table 1 lists the characteristics of AS-ODNs that need to be incorporated into their design and use.

AS-ODNs have several potential sites of action. AS-ODNs inhibit translation by hybridizing to the specific mRNA that they are designed for and the hybridization prevents either ribosomal assembly or ribosomal sliding along the mRNA [16]. This kind of action assumes that AS-ODNs are acting in the cytosol and do not affect measurable mRNA levels. Indeed, there are several articles reporting antisense effects without detectable change in target mRNA levels [6].

The general principles we follow in the design of the antisense are described in the following protocol.

Table 1. Characteristics of ideal antisense ODNs

1.	The DNA sequence is specific and unique
2.	Uptake into cells is efficient
3.	The effect in cells is stable (for long-term treatment) or transient (for short-term treatment)
4.	There is no non-specific binding to protein
5.	Hybridization of the ODN is specific for the target DNA
6.	The targeted protein and/or mRNA level is reduced
7.	The ODN is not toxic
8.	No inflammatory or immune response is induced
9.	The ODN is more effective than appropriate sense and mismatch ODN controls

Protocol for design of AS-ODN

Check GenBank for mRNA sequence of the target protein in the species to be studied

If there is more than one lab cloning the same protein, compare the homology of different reports. These sequences will point out the controversial bases, which can be due to natural mutations in variant strains of the same species or sequencing errors. Try to avoid these debatable regions. Target the ODN to the identical regions, which ensures reliability of the sequence being used. The best targets for designing effective AS-ODNs are the 5' cap region, the AUG translation initiation codon and the 3' untranslated region of the mRNA [17–19].

Choose the length of the antisense

The AS-ODNs we are using are 15–20 bases long, but longer or full-length cDNA in the antisense direction is used in plasmid or viral vectors. When designing antisense molecules, one has to consider two antagonistic factors: the affinity of oligonucleotide to its target sequence, which is dependent on the number and composition of complementary bases, and the availability of the target sequence, which is dependent on the folding of the mRNA molecule [20–22].

Check if antisense sequence is unique using Blast Search

Antisense sequence should be unique and specific for the target mRNA.

Avoid antisense with complicated secondary structure and self-dimerizing

Complicated secondary structures like loops and hairpins in the antisense sequence and self-dimerization prevent degradation, but also make hybridization with target mRNA more difficult. We are using phosphorothioated antisense which is much more resistant to degradation than natural phosphodiester, therefore more important is to facilitate interaction with target mRNA. To check the presence of the secondary structure we use program for designing PCR primers.

Stability of ODNs

Oligonucleotides in their natural form as phosphodiesters are subject to rapid degradation in the blood, intracellular fluid or cerebrospinal fluid by exo- and endonucleases.

The most widely used modified ODNs are phosphorothioates, where one of the oxygen atoms in the phosphodiester bond between nucleotides is replaced with a sulfur atom. These phosphorothioate ODNs have greater stability in biological fluids than normal oligos. The half-life of a 15-mer phosphorothioate ODN is 9 h in human serum, 14 h in tissue culture medium and 19 h in cerebrospinal fluid [23].

Cellular uptake of ODNs

In order to hybridize with the target mRNA, AS-ODNs have to cross the cell membrane. Saturable uptake of ODNs reaches a plateau within hours, occurs rapidly and, depending on the cells, can be efficient [24–26]. Uptake is faster for shorter ODNs than for longer ones.

Pharmacology of AS-ODNs

Antisense inhibition can be considered pharmacologically as a drug-receptor interaction, where the oligonucleotide is the drug and the target sequence is the receptor. For binding between the two affinity is provided by hydrogen bonding between the Watson-Crick base pairs and base stacking in the double helix that is formed. In order to achieve pharmacological activity, a minimum number of 12–15 bases can provide the minimum level of affinity [26].

Increasing the length of the ODN should result in higher level of specificity, but it also decreases its uptake into cells [23]. With viral vectors, however, the length problem is overcome because the virus enters cells by binding to viral receptors on cell membranes. Therefore, in a viral vector a full-length DNA-antisense sequence can be used. The mechanism of action of antisense DNA differs from that of the antisense ODN. The antisense DNA produces an antisense mRNA that competes negatively with cell native mRNA.

Toxicity of AS-ODNs

AS-ODNs can inhibit protein synthesis in cultured cells in nmol/L doses. The therapeutic window for AS-ODNs is rather narrow: when testing for the optimal dose, small increments in the high nmol/L range should be tested [26, 27]. High concentrations may produce non-specific binding to cytosolic proteins and give misleading results.

Phosphodiester ODNs are degraded to their naturally occurring nucleotide building blocks relatively quickly, therefore no toxic reaction is expected from even high doses of phosphodiester. Studies on phosphorothioated ODNs rats show that following intravenous injection, phosphorothioated ODNs are taken up from plasma mainly by the liver, fat and muscle tissues. Phosphorothioate ODNs are excreted through the urine in 3 days, mainly in their original form. An apparently mild increase in plasma lactate dehydrogenase (LDH) and, to a lesser extent, indicators of a possible transient liver toxicity with very high doses of phosphorothioated ODN was observed [28]. Whole new classes of ODN backbone modification are being developed to avoid the possible liver toxicity in humans with phosphorothioates [1–7, 27].

Delivery of AS as a naked ODNs

Direct injection of the AS-ODN to inhibit renin-angiotensin system has been used in the experiments described below [29–31]. For injections into the brain, ODNs appear to be very successful. In a number of studies, using different antisense ODNs, there has been efficient uptake and effective reduction in protein as well as inhibition of the physiological parameters studied. Uptake is so efficient that one difficulty with intracerebroventricular injections is that the ODN tends to be taken up close to the site of injection and not to spread to other parts of the brain. While this has little impact for hypertension therapy, it is an important consideration in antisense strategies for the treatment of brain diseases, such as Parkinson's disease, thalamic pain, Alzheimer's disease and gliomas.

ODNs and liposomes

Liposomes which are self-assembling particles of bilipid layers have been used for encapsulating antisense ODN for delivery in blood and cell culture [32–35]. Antisense, directed to angiotensinogen mRNA in liposomes, has had successful results.

Tomita et al. [34] used liposome encapsulation of angiotensinogen antisense and a Sendai virus injected into the portal vein of spontaneously hypertensive rats (SHR). Blood pressure decreased for several days. However, they did not compare their results with the effects of naked ODN. Wielbo et al. [35] compared liposome-encapsulated antisense and naked ODN given intra-arterially. They found that liposome encapsulation was effective, whereas naked ODN was not, under the same conditions. Twenty-four h after injection of 50 μg of liposome-encapsulated antisense ODN, blood pressure decreased by 25 mm Hg. Neither empty liposome nor liposome-encapsulated scrambled ODN nor AS-ODN alone

had a significant effect on blood pressure. Confocal microscopy of rat liver tissue 1 h after intra-arterial injection of 50 μg of not encapsulated fluorescein isothiocyanate (FITC) AS, or liposome-encapsulated FITC-conjugated AS, showed intense fluorescence in liver tissue sinusoids with the liposome-encapsulated ODN. Levels of protein (angiotensinogen and angiotensin II in the plasma) were significantly reduced in the liposome-encapsulated ODN group. Antisense alone, lipids alone, and scrambled ODN in liposomes had no effect on protein levels [35]. The short, single-stranded AS-ODNs are not in fact encapsulated but complexed with bilamellar vesicles by electrostatic interactions. Liposome development with cationic lipids also allows high transfection efficiency of plasmid DNA.

Plasmid vectors

Partial or full-length cDNA of the gene of interest can be subcloned in antisense orientation under chosen promoter in a plasmid vector. Antisense mRNA is then expressed and competes with host sense mRNA for translation machinery. Plasmid vectors cause a relatively small immune response and can deliver long DNA sequences. Current limitations are mainly a low efficiency of gene transfer *in vivo* and poor long-term expression.

We constructed plasmid vector containing part of the AT_1-R sequence in antisense orientation under the CMV promoter. This plasmid was tested for AT_1 receptor inhibition *in vitro*, using NG108-15 [36] and vascular smooth muscle cells (VSMC) [37]. The NG108-15 cells had a significant ($p < 0.01$) decrease in angiotensin II AT_1 receptors number comparing with the control cells. No effect was seen on the AT_2 receptors. Rat VSMC transfected with the plasmid showed decrease in AT_1-R mRNA and reduced calcium response to angiotensin II stimulation [37].

Also, we subcloned full-length angiotensinogen (AGT) cDNA in antisense orientation under the CMV promoter. This plasmid vector was used for transient transfection of hepatoma cells H4-IIE. Antisense expression was detected from 2 h after transfection and caused 50% reduction in AGT level secreted by the cells [38]. AGT-AS plasmid injected i.v. (3 mg/kg body wt) to adult SHRs caused a decrease in the blood pressure to a maximum of −22 mm Hg, as compared to sense or saline-injected animals, lasting up to 6 days [39].

For the local effect the most frequently used route of administration for plasmid DNA is probably intramuscular injection.

Generally, use of plasmid DNA is considered to be safer than viral vectors but expression is less efficient and shorter lasting, therefore, is useful when the transient effect is sufficient.

Viral vectors for antisense DNA delivery

Perfect viral vector

There are several viruses which have been tested for gene delivery, and each has its advantages, but does not fit perfectly to the description of the 'ideal viral vector'. To be the perfect vector, a virus should fulfill all of the following criteria listed in Table 2.

Retroviruses

These have been used primarily because of their high efficiency in delivering genes to dividing cells [40]. Retroviruses permit insertion and stable integration of single copy genes. Although effective in cell culture systems, they randomly integrate into the genome, which raises concerns about their safety for practical use *in vivo*. Because retroviruses can only act in dividing cells, they are ideal for tumor therapy but less desirable where other cells are dividing that need to be protected.

In hypertension research, retroviruses are being investigated in treating developing SHR. Our colleagues at the University of Florida delivered retrovirus vector (LNSV) containing an antisense DNA to AT_{1-B} receptor mRNA [41]. Injections in the heart of 5 day old SHR resulted in effective, long-term inhibition of AT_1 receptor mRNA and significant inhibition of the development of hypertension. Several measures indicated that the treatment reduced responsiveness of AT_1-R in vessels to angiotensin II stimulation [41]. Problems could arise, however from incorporating the virus into the germ line.

Adenoviruses

Adenovirus vectors have been tested successfully in their natural host cells, the respiratory endothelia, as well as other tissues such as vascular smooth and striated muscle, and brain [42–44].

Adenovirus is a double-stranded DNA with 2700 distinct adenoviral gene products. It infects many mammalian cell types because most cells have membrane receptors. The viruses enter the cell by receptor-induced endocytosis and translocate to the nucleus. Most adeno-virus vectors in their current form are episomal, that is, they do not integrate into the host DNA. They provide high levels of expression, but the episomal DNA will invariably become inactive after some time. In some species, e.g. mice, this time may be long, relative to their life span, but in humans it is a limitation of the virus as a vector. Repeated infections result in an inflammatory response with consequent tissue damage. This is because the adenovirus expresses genes that lead to immune cell attacks. This further limitation makes current recombinant adenovirus unsuitable for long-term treatment and several gene therapy trials using adenovirus vectors have failed to produce acceptable results. Preliminary studies with adenovirus vectors for delivery of AT_1-R mRNA antisense have been tested in rats and reduced developing hypertension in SHR [45]. The adenovirus is easy to produce and therefore useful for animal studies of mechanisms. However, the adenovirus as a vector has too many limitations at present to be successful in human gene therapy. Further engineering of the adenovirus may eventually avoid these limitations. Promising results have been obtained by Burcin *et al.* [46] who used adenovirus devoid of all viral coding sequences [47].

Adeno-associated virus (AAV)

The AAV has been gaining attention because its safety and efficiency [48]. It has been successfully used for delivering antisense RNA against alpha-globin [49] and HIV LTR [50], and it is our vector of choice for delivering antisense targeted to the renin-angiotensin system in hypertensive animal models.

AAV is a parvovirus, discovered as a contamination of adenoviral stocks. It is widespread (it is estimated that antibodies are present in 85% of people in the USA) and has not been linked to any disease. Its replication is dependent on the presence of a helper virus, such as adenovirus or herpes virus. Five serotypes have been isolated, of which AAV-2 is the best characterized. AAV has a single-stranded linear DNA which is encapsidated into capsid proteins VP_1, VP_2 and VP_3 to form an icosahedral virion of 20–24 nm in diameter [48].

The AAV DNA is approximately 4.7 kb long. It contains two open reading frames and is flanked by two inverted terminal repeats (TRs). There are two major genes in the AAV genome: *rep* and *cap*. *Rep* codes for proteins responsible for viral replications, whereas *cap* codes for capsid proteins VP_{1-3}. Each TR forms a T-shaped hairpin structure. These ter-

Table 2. Features of the perfect viral vector

1.	Is safe, so if it is known to cause disease, it must be re-engineered to be harmless
2.	Does not elicit an immune or inflammatory response
3.	Does not integrate into the genome randomly, as this would risk disrupting other cellular genes and mutagenesis
4.	Is replication-deficient so that it will not spread to other tissues or infect other individuals
5.	It delivers a defined gene copy number into each infected cell
6.	Is efficiently taken up by the target tissue, therefore, infects the target cells with high efficiency
7.	Has high capacity so it can accommodate the gene of interest, along with its regulatory sequences
8.	The recombinant DNA is packaged with high efficiency into the viral capsid
9.	Is easy to manipulate and produce in pure form

minal repeats are the only *cis*-components of the AAV necessary for packaging. Therefore, the AAV can be used as a vector with all viral coding sequences removed and replaced by the cassette of genes for delivery. Three viral promoters have been identified and named p5, p19 and p40, according to their map position. Transcription from p5 and p19 results in production of *rep* proteins, while transcription from p40 produces the capsid proteins [48].

Upon infection of a human cell, the wild-type AAV integrates into the q-arm of chromosome 19 [51, 52]. Although chromosomal integration requires the terminal repeats, the viral components responsible for site-specific integration have been recently targeted to the *rep* proteins [53]. With no helper virus present, AAV infection remains latent indefinitely. Upon super-infection of the cell with helper virus, the AAV genome is excised, replicated, packaged into virions and released to the extracellular fluid. This fact is the basis of recombinant AAV production for research.

Several factors prompted researchers to study the possibility of using recombinant AAV as an expression vector. Benefits and drawbacks of using AAV are listed in Table 3.

The advantages, particularly its safety, make AAV appear to be one of the best candidates for delivery of genes for long-term therapy. For more powerful expression native promoter can be replaced by a stronger one, like the cytomegalovirus (CMV) promoter. For expression in a particular tissue, there is an option of local injection or use of tissue-specific promoters. Recently, Flotte *et al.* [55] have established gene therapy Phase I trials for cystic fibrosis using AAV gene delivery in patients.

The general concept for antisense gene delivery in the AAV vector and the steps involved are shown in Fig. 1. To illustrate these steps, a brief description is given that is applicable to the renin-angiotensin system. Further details are presented in the reference [55].

The first step toward obtaining the recombinant AAV, is to construct a plasmid containing AAV terminal repeats (pAAV). Example below refers to AT1R-AS.

Method for preparation of pAAV-AT$_1$R-AS

The 749 bp fragment of the AT$_1$-R cDNA (−183 to 566) was amplified using polymerase chain reaction (PCR) and ligated to an AAV-derived vector (Fig. 2) in the antisense orientation,

Table 3. Features of AAV as a vector

Benefits	Drawbacks
1. Non-pathogenic	1. Capacity up to 4.4 kb
2. Very broad host range [54]	2. Complex packaging requires
3. Virus sequences limited to ITRs (290 bp), therefore, does not evolve an inflammatory response	helper virus and helper plasmids with *rep* and *cap* genes
4. Long-lasting expression	3. Multistep purification method

in place of *gfp*. The resulting plasmid vector (pAAV-AT$_1$R-AS) contained adeno-associated virus terminal repeats (TR), a cytomegalovirus promoter (Pcmv), the DNA encoding AT$_1$ receptor mRNA in the antisense orientation and a *neomycin* resistance gene (*neo^r*) (Fig. 3). *Escherichia coli* bacteria (Sure II, Stratagene) were transformed with the plasmid according to the manufacturer protocol. The plasmid DNA was purified on CsCl gradient.

We constructed plasmids for both AT$_1$ receptor antisense and angiotensinogen antisense in the AAV-derived expression vector. Initially we used a plasmid containing AAV genome and 750 bp cDNA inserted into the AAV in the antisense direction downstream from the AAV promoter. The NG108-15 cells or hepatoma H4 cells were transfected with AT$_1$-R-AS or AGT-AS plasmid, respectively, using lipofectamine [56, 57]. In both cases, there were significant reductions in the appropriate proteins, namely AT$_1$-R and angiotensinogen. To test that the cells expressed AAV we used the *rep* gene product as a marker. Immunocytochemical staining with a rep protein antibody showed that the majority of cells in culture expressed the vector. A further development of the AAV was the insertion of more powerful and specific promoters than the p40 promoter. AAV with CMV promoter and *neomycin* resistance (*neo^r*) gene as a selectable marker is now being used in our current experiments. The AAV cassette contained a marker, either the *gfp* gene which encodes the green fluorescent protein (Fig. 2, ref. [58]) or *lacZ* gene [59]. NG108-15 cells transfected with AAV plasmid containing the *gfp* and *neo^r* genes were selected by antibiotic, G418 (600 μg/ml), and the selected clones viewed for GFP expression. Very few cells died during selection. Two weeks after transfection all of the cells were expressing GFP. The transfection efficiency of this pAAV-*gfp* construct in different cell lines, including ATt20 (mouse pituitary cells), L929 (mouse fibroblasts), HEK293 (human embryonic kidney cells) and NG108-15 was over 50% [60].

Packaging rAAV

To prepare recombinant AAV, Human Embryonic Kidney (HEK293) cells are transfected with plasmid vector containing gene of interest in antisense orientation and AAV terminal repeats (pAAV-AS), together with helper plasmid delivering *rep* and *cap* genes (necessary for AAV replication) in *trans* using calcium phosphate method. Eight h after transfection, helper virus is added at a multiplicity of infection (MOI) of 5. Some methods use plasmids-only packaging, without the helper virus. There are few methods used to package and purify the rAAV. Details are described in refs [61, 62]. The titer of the virus is assayed using HEK293 cells, wild type AAV and adenovirus.

In our studies, recombinant AAV-AT$_1$R-AS was tested for AT$_1$ receptor inhibition *in vitro*, using vascular smooth muscle cells [37]. Transduced cells, without G418 selection, expressed
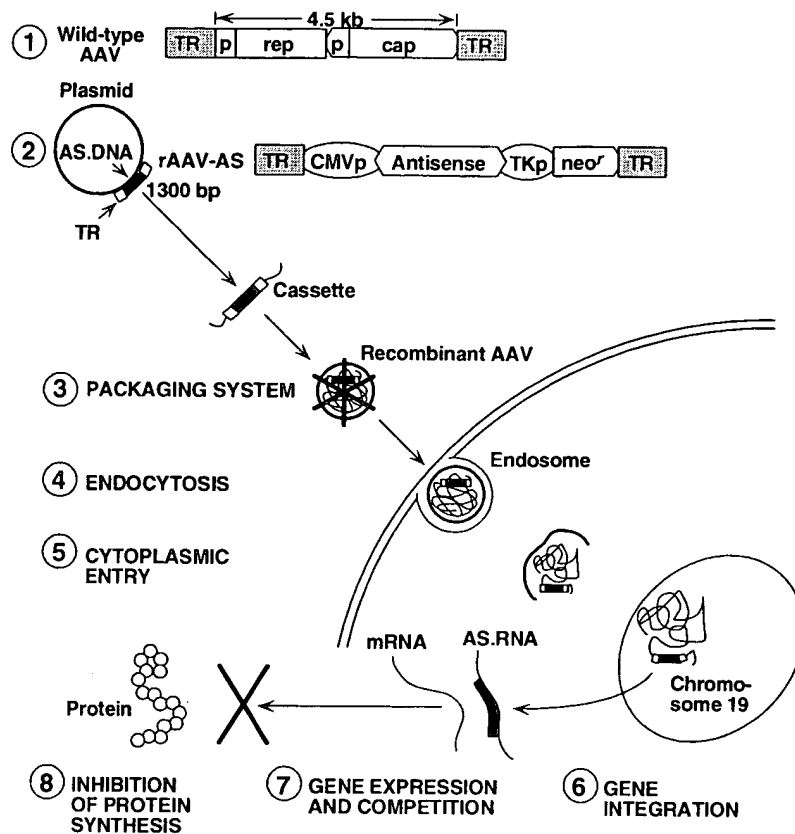
150

Fig. 1. Gene delivery of AS DNA with adeno-associated viral vector (AAV). 1. Wild-type AAV (simplified): terminal repeats (TR), native promoters (p), genes necessary for replication (rep) and capsid (cap). 2. AAV-based plasmid: The plasmid contains the terminal repeats (TR) characteristic of AAV. A cassette contains CMV promoter-driven antisense and TK promoter-driven neo^r gene for selection by G418 (geneticin). 3. Virus packaging: The packaging cell line is transfected with AAV-based plasmid and helper plasmid with *rep* and *cap* genes, and transduced with adenovirus as a helper virus. 4. Endocytosis: The viral vector fuses with the cell membrane by binding to adhesion molecules and becomes an endosome within the bilipid layer. 5. Cytoplasmic entry: The vesicle "opens" in the cytoplasm, releasing the vector that is transported to, and enters the nucleus. 6. Gene integration: AAV integrates with chromosome 19. It is not known if the addition of foreign DNA interferes with this integration. 7. Gene expression and competition: Genomic DNA in chromosome 19 produces an antisense RNA (AS-RNA). This competes with the natural mRNA and decreases its translation. 8. Inhibition of protein synthesis: A binding of antisense RNA to the mRNA prevents sliding through the ribosomal assembly to produce protein. The result of this gene delivery system should be reduction in the amount of protein, specifically targeted by the antisense.

the transgene for at least 8 weeks, had a decreased number of AT$_1$ receptors and reduced calcium response to angiotensin II stimulation.

Expression *in vivo* was tested first by direct injection into the brain. An AAV with the arginine vasopressin promoter (AVP) to drive a *lacZ* reporter gene was constructed. The vector expressed β-galactosidase in neurons of the paraventricular nucleus and supraoptic nucleus. The expression was in magnocellular cells which normally express AVP [59]. The expression was observed at 1 day, 1 week and after 1 month with no diminution of signal. This is an example of how AAV can be developed for specific tissue and/or cell gene expression and shows that AAV vectors can deliver foreign genes into adult brain for long periods of time.

Next, to test for effectiveness *in vivo*, rAAV-AT$_1$R-AS was microinfused into the lateral ventricles of adult male SHR. Control rats received AAV with *gfp* reporter gene but without the AS gene ('mock' vector) in vehicle which was artificial cerebrospinal fluid. Blood pressure was measured by tailcuff method. There was a significant decrease in systolic blood pressure (SBP) in one group of rats that received the rAAV-AS vector. No effect was observed in the controls. SBP decreased by 23 ± 2 mm Hg in the first week after administration. This drop in blood pressure was prolonged in 4 rats for 9 weeks, whereas controls had no reduction in blood pressure [63]. This was considerably longer than the longest effect observed with AS-ODN. Recombinant AAV-*gfp* expression in hypothalamus of the control rat group was de-
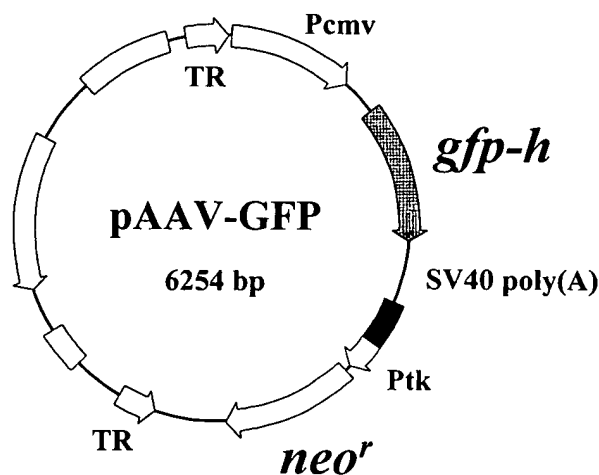
Fig. 2. Schematic diagram of the recombinant AAV vector containing the
gfp gene. In the recombinant AAV-gfp vector almost all of the parental wild-
type AAV genome (except the terminal repeats – TR) has been deleted and
replaced with gfp, the gene encoding Aequorea Victoria green fluorescent
protein gene, driven by a CMV promoter (Pcmv). The neomycin resistance
(neoʳ) gene is under the control of thymidine kinase promoter (Ptk). The
gfp serves as a reporter gene in vitro or in vivo, and the neoʳ serves for se-
lection in vitro.

tectable by RT-nested PCR 11 months after injection. Further,
intra-cardiac injection of rAAV-AS in SHR, significantly
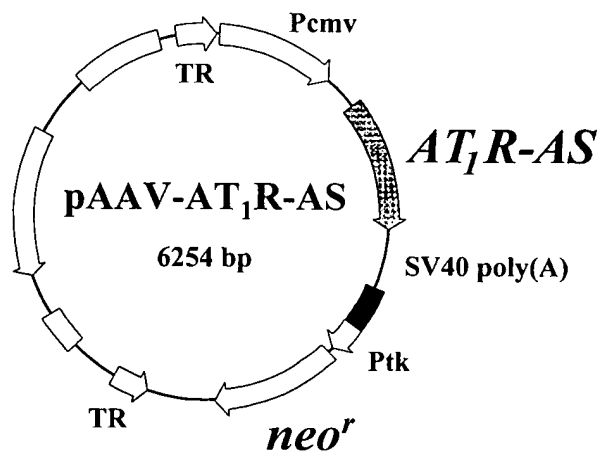reduced blood pressure and slowed the development of hy-

Fig. 3. Plasmid vector pAAV-AT₁R-AS contains a 750 bp fragment of the
AT₁ₐ receptor cDNA in antisense orientation. Abbreviations: TR – AAV
terminal repeat; Pcmv – human cytomegalovirus early promoter; SV40 poly
(A) – polyadenylation signal from simian virus; Ptk – thymidine kinase
promoter; neoʳ– neomycin phosphotransferase gene from Tn5. Other pro-
moters have been substituted for CMV, including the arginine vasopressin
(AVP), neuron specific enolase (NSE) and glial fibrillary acid protein (GFAP)
promoters.

pertension for several weeks [63, 64]. This result demon-
strates that rAAV-AS in a single application, is effective in
chronically inhibiting renin-angiotensin system. This encour-
ages further research on gene regulation in cardiovascular dis-
eases and to explore the most effective routes of delivery
applicable to humans.

Acknowledgements

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ANTISENSE STRATEGIES IN NEUROBIOLOGY

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Abstract—The use of antisense oligodeoxynucleotides, targeted to the transcripts encoding biologically active proteins in the nervous system, provides a novel and highly selective means to further our understanding of the function of these proteins. Recent studies of these agents also suggest the possibility of their being used therapeutically for a variety of diseases involving neuronal tissue. In this paper we review studies showing the *in vitro* and *in vivo* effects of antisense oligodeoxynucleotides as they relate to neurobiological functions. Particular attention is paid to the behavioral and biochemical effects of antisense oligodeoxynucleotides directed to the various subtypes of receptors for the neurotransmitter dopamine. An example is also provided showing the effects of a plasmid vector expressing an antisense RNA targeted to the calmodulin mRNAs in the PC12 pheochromocytoma cell line. The advantages of antisense oligodeoxynucleotides over traditional pharmacological treatments are assessed, and the advantages of using vectors encoding antisense RNA over the use of antisense oligodeoxynucleotides are also considered. We also describe the criteria that should be used in designing antisense oligodeoxynucleotides and several controls that should be employed to assure their specificity of action. © 1997 Elsevier Science Ltd

INTRODUCTION

Antisense technology, the use of oligonucleotides to inhibit gene expression through sequence-specific hybridization, has gained increasing acceptance for studying fundamental problems in neurobiology and for studying, and possibly treating, neurobiological diseases. The concept upon which this technology is based is fundamentally different from that used in the past. The classical pharmacological approach is to inhibit the function of biologically active proteins (e.g. neurotransmitter receptors) by interfering with the interaction between these proteins and the endogenous activators (e.g. neurotransmitters). The antisense strategy, in contrast, is to inhibit the synthesis of these proteins by preventing their expression at the level of RNA or DNA. Several different strategies based on the antisense approach to block gene expression have been developed: antisense oligodeoxynucleotides, which target the messenger RNA encoded by an individual gene; antisense polyribonucleotides (antisense RNA), which also target the mRNA but which are produced *in situ* through the use of DNA sequences

encoding the antisense RNA; the triplex "antigene" strategy, which utilizes oligonucleotides to target DNA instead of RNA; and the ribozyme strategy, where the antisense RNA has the additional ability to catalyse the metabolism of the duplex formed. The inhibition of gene expression at the DNA level has also been achieved using a "sense" strategy, wherein DNA sequences to which transcription activating factors bind are introduced into the cell. The transcription activating factors thereby become unavailable to bind to DNA and to activate gene transcription.

Among the various strategies, the use of antisense oligodeoxynucleotides and antisense RNA to inhibit gene expression have been most widely used and have shown the greatest promise as tools to study a wide variety of biological problems (Cohen, 1989; Helene and Toulme, 1990; Baserga and Denhardt, 1992; Crooke, 1992; Stein and Cheng, 1993; Knorre *et al.*, 1994; Murray, 1994; Crooke, 1995a,b; De Mesmaeker *et al.*, 1995). The antisense oligodeoxynucleotide approach has been used: (a) in virology to inhibit the replication of HIV and other viral agents (Cohen, 1991; Goodchild, 1991; Malcolm *et al.*, 1992); (b) in oncology to inhibit the proliferation of malignant cells both *in vitro* and *in vivo* (Dolnick, 1991; Neckers *et*

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al., 1992a; Ferrari *et al.*, 1994; Pierga and Magdelenat, 1994; Schwab *et al.*, 1994); (c) in cardiology to inhibit the proliferation of the arterial intima (Simons *et al.*, 1992; Simons and Rosenberg, 1992); (d) in endocrinology to study the various stages of stimulus-response coupling in endocrine cells and in hormone target cells; and (e) in neuropharmacology to inhibit the function of a variety of neuroreceptors and other neuronal proteins (see Tables 1–3). Moreover, clinical trials are already being carried out in the U.S.A. with antisense compounds, some of which are intended to limit the development of AIDS, genital warts, kidney transplant rejection, rheumatoid arthritis and other autoimmune diseases, and chronic myelogenous leukemia (Gura, 1995). This review will focus on the *in vitro* and *in vivo* applications of antisense oligodeoxynucleotides and vector-generated antisense RNA as tools to study neurobiological events and as potential therapeutic agents to treat neurological and neuropsychiatric disorders.

Antisense oligodeoxynucleotides

Antisense oligodeoxynucleotides are short (usually 15–20 bp), single stranded DNA sequences, which are complementary to a portion of an RNA or a DNA, and thereby form complexes with the target RNA or DNA through Watson–Crick interactions. During the past few years they have been extensively applied in many areas of biological research because they can be readily synthesized *in vitro*, and can be used to inhibit and study the function of specific genes by laboratories that do not have extensive molecular biological facilities.

The major issues of concern in the use of antisense oligodeoxynucleotides in neurobiology are similar to those encountered in other fields: these include the penetration of oligodeoxynucleotides into tissues, the stability of antisense oligodeoxynucleotides once inside the cell, and their efficacy and specificity of action. In many neurobiological experiments there is the additional problem of the penetration of antisense oligodeoxynucleotides across the blood–brain barrier (Agrawal *et al.*, 1995).

Many reviews have already been devoted to the problems of the chemical structure, principles of design, and mechanisms of action of antisense oligodeoxynucleotides (Uhlmann and Peyman, 1990; Cohen, 1991; Crooke, 1992; Leonetti *et al.*, 1993; Milligan *et al.*, 1993). Therefore, these issues will only be touched upon here. Our major purpose will be to review briefly those aspects that are most relevant to neurobiology.

Structural modifications of antisense oligodeoxynucleotides

In order to increase the biological half-life and efficacy of the "natural" antisense oligodeoxynucleotides, several chemical modifications have been made in their structure. These include changes in the phosphodiester backbone, in the deoxyribose sugar, and in the heterocyclic base moieties (reviewed in Uhlmann and Peyman, 1990; Cohen, 1991; Crooke, 1992). Among the most widely used modified oligodeoxynucleotides in neurobiology are the phosphorothioates, in which one of the non-bridging oxygen atoms in the conventional phosphodiester bond has been replaced with a sulphur atom. The phosphorothioate oligodeoxynucleotides are extremely resistant to the action of endo- and exonucleases, they are water soluble, and they penetrate the cells with a relatively high efficiency. They are superior to the methylphosphonates, another commonly employed derivative of the oligodeoxynucleotides, because unlike the methylphosphonates, the phosphorothioate:mRNA hybrid is a substrate for the enzyme RNase H.

Several other strategies have been developed to increase the stability and cellular penetration of the oligodeoxynucleotides (reviewed in Uhlmann and Peyman, 1990; Crooke, 1992). In one of the strategies the oligodeoxynucleotides are conjugated at the 5' or 3' end with lipophilic or other groups (e.g. thiolinkers, biotin, cholesterol) in order to improve cellular uptake and nuclease resistance. Attempts have also been made to enhance the cellular penetration of oligodeoxynucleotides by utilizing receptor-mediated uptake systems (Bonfils *et al.*, 1992; Budker *et al.*, 1992; Citro *et al.*, 1992; Juliano and Akhtar, 1992) or by complexing oligodeoxynucleotides to cationic lipids, such as N-[-1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). Cationic lipids can greatly increase the cellular uptake both of oligodeoxynucleotides and expression vectors producing antisense RNA, and several improvements in their structure have been made, including cationic lipids that are effective in serum-containing media (e.g. 1,2-dioleoyl-3-trimethylammonium-propane; DOTAP).

PRINCIPLES OF DESIGN AND MECHANISM OF ACTION
OF ANTISENSE OLIGODEOXYNUCLEOTIDES

Although the optimal target site of mRNA is not obvious (Laptev *et al.*, 1994), antisense oligodeoxynucleotides have been most often targeted to sites

Table 1. Effects of antisense oligodeoxynucleotides on the level and function of receptors and non-receptor proteins *in vitro* in neuronal tissue

Gene product	Cell type	AS	Conc. (μM) [EC₅₀ (μM)]	Treatment time	Function	Target protein	Target mRNA	Reference
Neuronal receptors								
ACh nicotinic	Embryonic neurons	N	10	2 days	Decrease channel opening			Yu et al., 1993
ACh m₃ muscarinic	Cerebellar granule neurons	N	258	6 days	Decrease inhibition of cAMP content	Decrease		Holopainen and Wojcik, 1993
Angiotensin AT1	Neuroblastoma N1E–115 cells	S	5	1 day		Decrease		Sakai et al., 1994
Dopamine D₂	Pituitary cells	S	2	7 days	Loss of response	Decrease		Valerio et al., 1994
Dopamine D₂	Retinoblastoma WERI–27 cells	S	1	2 days		Decrease	Decrease	Silvia et al., 1994
GABAₐ	Cerebellar granule neurons	N	25 [5]	6 days	Decrease inhibition of cAMP content			Holopainen and Wojcik, 1993
Progesterone	T47D cells	N	1.5–12 [12]	2 days		Decrease		Pollio et al., 1993
Substance P neurokinin 1	Astrocytoma U-87 MG cells	S	30	2 days	Decrease Ca²⁺ influx	Decrease		Ogo et al., 1994
Non-receptor proteins								
Amyloid precursor protein	PC12 cells	S	10–20 μg/ml	7 days	Decrease cell body size and neurite outgrowth	Decrease		Majocha et al., 1994
Brain-derived neurotrophic factor	Cultured dorsal root ganglion (DRG cells)	N	0.5–10	3 days	Decrease in neuronal survival	Decrease		Acheson et al., 1995
bFGF	Glioblastoma U87 MG cells	S	5	6 days	Inhibit proliferation	Decrease		Murphy et al., 1992
Calmodulin	PC12 cells	S	12	5 days	Inhibit proliferation	Decrease		Zhang et al., 1994a
c-fos and c-jun	Pituitary AtT–20 cells	N	10	23 h	Inhibit Il-1-induced beta-endorphin release			Fagarason et al., 1990
c-jun	PC12 cells	S	2	10 days	Induce neurite outgrowth	Decrease		Schlingensiepen et al., 1993
c-myc	Neuroepithelioma CHP100 cells	N	100	17 h	Inhibit growth	Decrease		Rosolen et al., 1991
Dynamin	Hippocampal neurons	N	50 [12.5]	2 days	Inhibit neurite outgrowth	Decrease		Torre et al., 1994
GAP-43	PC12 cells	N	75	1 day	Inhibit neurite outgrowth			Jap Tjoen San et al., 1992
Gephyrin	Spinal neurons	S	5	6 days	Prevent formation of GlyR clusters	Decrease		Kirsch et al., 1993
jun-b	PC12 cells	S	2	10 days	Inhibit neurite outgrowth	Decrease		Schlingensiepen et al., 1993
Peripherin	PC12 cells	N	0.2	42 days	Inhibit neurite outgrowth			Troy et al., 1992
Phospholipase A2	CHO cells	N	50	1 day	Decrease arachidonic acid	Decrease		Vial and Piomelli, 1995
SNAP-25	Cortical neurons and PC12 cells	S	2 [0.5]	10 days	Inhibit neurite outgrowth	Decrease		Osen-Sand et al., 1993
Tau	Cerebellar neurons	N	3–50	1 day	Inhibit neurite outgrowth	Decrease		Caceres and Kosik, 1990
Tau	PC12 cells	N	150	3 days	Inhibit neurite outgrowth	Decrease		Hanemaaijer and Ginzburg, 1991
Tau	Cerebellar neurons	N	25	1h	Inhibit neurotoxicity	Decrease		Pizzi et al., 1995
Tubulin	PC12 cells	N	12.5 125 [50]	1.5 days	Inhibit neurite outgrowth	Decrease		Teichman-Weinberg et al., 1988
Vasopressin	Hypothalamic neurons	S	1–20	2 days		Decrease		Meeker et al., 1995
Vimentin	Neuroblastoma NB2a/d1 cells		25	1 day	Inhibit neurite initiation	Decrease		Shea et al., 1993

AS, antisense oligodeoxynucleotide; ACh, acetylcholine; GAP, growth-associated protein; SNAP, synaptosomal-associated protein; FGF, fibroblast growth factor; N, natural (unmodified) oligodeoxynucleotide; S, phosphorothioate oligodeoxynucleotide; GlyR, glycine receptor.

Table 2. Effects of antisense oligodeoxynucleotides on the level and function of receptors and non-receptor proteins *in vivo* in neuronal tissue

Gene product	Species	Injection site	AS	Dose [EC$_{50}$]	Time	Function	Target protein	Target mRNA	Reference
						Neurotransmitter receptors			
ACh m$_1$ muscarinic	Rat	i.c.v.		5 µg/h	4 days		Decrease		Zang et al., 1994
Dopamine D$_1$	Mouse	i.c.v.	S	2.5 nmol [6 nmol]	7 days	Inhibit behavior			Zhang et al., 1994b
Dopamine D$_2$	Mouse	i.c.v.	S	2.5 nmol	Inhibit behavior			Decrease	Weiss et al., 1993
Dopamine D$_2$	Rat	i.c.v.	S	10 µg/h	4 days	Affect behavior	Decrease		Zhang and Creese, 1993
Dopamine D$_2$	Mouse	i.c.v.	S	2.5 nmol [0.7 nmol]	7 days	Affect behavior	Decrease	Decrease	Zhou et al., 1994
Dopamine D$_2$	Mouse	i.c.v.	S	2.5 nmol	2 days	Inhibit decrease body temperature			Weiss et al., 1996b
Dopamine D$_2$	Rat	S.N.	S	0.04 nmol	2.5 days	Induce behavior	Decrease		Silvia et al., 1994
Dopamine D$_2$	Mouse	i.c.v.	S	2.5 nmol		Affect behavior	Decrease		Qin et al., 1995
Dopamine D$_3$	Rat	i.c.v.	S	10 µg/h	5 days	Increase DA synthesis	Decrease		Nissbrandt et al., 1995
Dopamine D$_3$	Mouse	i.c.v.	S	2.5 nmol	7 days	Increase locomotion			Weiss et al., 1996a
NMDA-R1	Rat	i.c.v.	N	15 nmol	2 days	Reduce cell death	Decrease	No change	Wahlestedt et al., 1993a
NMDA-R1	Rat	i.c.v.	N	15 nmol/5 µl	14 days	Protect brain injury			Sun and Faden, 1995
Serotonin 5-ht6	Rat	i.c.v.	S	12 and 24 µg	6 days	Induce yawning, stretching and chewing	Decrease		Bourson et al., 1995
						Peptide receptors			
Angiotensin AT1	Rat	i.c.v.	S	50 µg	1 day	Lower blood pressure	Decrease		Gyurko et al., 1993
Angiotensin AT1	Rat	i.c.v.	S	200 ng	3 days	Inhibit drinking	Decrease		Sakai et al., 1994
Angiotensin AT1	Rat	i.c.v.	S	50 µg	1 day	Inhibit drinking	Decrease		Meng et al., 1994
Angiotensin AT1	Rat	i.c.v.	S	50 µg	3 days		Decrease		Ambuhl et al., 1995
CCK$_B$	Mouse	i.c.v.	N	12.5 µg	3 days	Enhance antinociception			Vanderah et al., 1994
Neuropeptide Y-Y1	Rat	i.c.v.	N	50 µg	2 days	Display anxiety	Decrease		Wahlestedt et al., 1993b
Substance P neurokinin-1	Rat	i.c.v.		15 µg	7 days		Decrease		Ogo et al., 1994
TRH	Rat	i.t.	N	100 µg	3 days	Reduce blood pressure			Suzuki et al., 1995
Vasopressin V1	Rat	Septum	S	0.5 µg/h	4 days	Reduce anxiety-related behavior	Decrease	Increase	Landgraf et al., 1995
						Opioid receptors			
δ	Mouse	i.t.	N	1 and 5 µg	6 days	Inhibit analgesia	Decrease		Standifer et al., 1994
δ	Mouse	i.t.	N/S	1.6–163 pmol	3 days	Inhibit antinociception			Tseng et al., 1994
δ	Mouse	i.c.v.	N	25 µg	3 days	Inhibit antinociception			Lai et al., 1994
δ	Rat	i.c.v.	N	100 µg			Decrease		Cha et al., 1995
δ	Mouse	i.t.	S	1 µg	3 days	Inhibit antinociception			Narita and Tseng, 1995
κ	Rat	i.c.v.	S	20 µg	6 days	Inhibit analgesia			Adams et al., 1994
	Rat	PAG	N	10 µg	6 days	Inhibit analgesia			Rossi et al., 1994
µ	Rat	PAG	N	10 µg	6 days	Inhibit analgesia			Rossi et al., 1995
µ	Rat	i.c.v.	N	20 µg	6 days	Inhibit analgesia			Chen et al., 1995

Table 2—*continued*

Gene product	Species	Injection site	AS	Dose [EC₅₀]	Time	Function	Target protein	Target mRNA	Reference
μ and δ	Rat	i.c.v.	S	30 μg	6 days	Reduce growth hormone secretion			Idänpään-Heikkilä et al., 1995
μ, δ and κ	Mouse	i.c.v.	N	12.5 μg	3 days	Inhibit analgesia			Lai et al., 1995
Steroid receptors									
Estrogen	Rat	HYP	N	2 μg	6 h	Induce lordosis			McCarthy et al., 1993b
Oxytocin	Rat	HIP	S	400 ng	7 days	Reduce lordosis	Decrease		McCarthy et al., 1994b
Progesterone	Rat	HYP	N	400 ng	2 days	Reduce lordosis			Pollio et al., 1993
Progesterone	Rat	HYP	N	400 ng	12 h	Reduce lordosis	Decrease		Ogawa et al., 1994
Immediate-early genes									
c-fos	Rat	Striatum	S	0.5, 1 and 2 nmol	10 h		Decrease		Chiasson et al., 1992
c-fos	Rat	Striatum	S	2 nmol	5 h	Induce rotation	Decrease		Sommer et al., 1993
c-fos	Rat	Striatum	S	9 μg	10 h	Induce rotation	Decrease		Dragunow et al., 1993
c-fos	Rat	Medulla	S	0.2 nmol	6 h	Decrease blood pressure	Decrease		Suzuki et al., 1994
c-fos	Rat	N.A.	S	10 nmol	8 h	Inhibit locomotion			Heilig et al., 1993
c-fos	Rat	Spinal cord	S	0.375 nmol	6 h		Decrease		Gillardon et al., 1994
c-jun	Rat	HIP	S	4 nmol	10 h	Inhibit learning			Tischmeyer et al., 1994
Non-receptor proteins									
Angiotensinogen	Rat	i.c.v.	S	50 μg	1 day	Lower blood pressure	Decrease		Wielbo et al., 1995
CRH	Rat	i.c.v.	S	30 μg	1.5 days	Reduce anxiety	Decrease	Decrease	Skutella et al., 1994a
GAD65 and GAD67	Rat	HYP, central grey	N;S	250 and 500 ng	4 days	Reduce lordosis	Decrease		McCarthy et al., 1994b
Gi2	Mouse	i.c.v.	S	12.5 μg	1 day	Inhibit anti-nociception			Raffa et al., 1994
Kinesin	Rabbit	Vitreous chamber of eye	S	0.1 μmol	20 h	Inhibit axonal transport	Decrease		Amaratunga et al., 1993
Neuropeptide-Y	Rat	i.c.v.	S	5 μg	7 days	Inhibit food intake and lower body weight	No change		Hulsey et al., 1995
Oxytocin	Rat	PVN	N	4 μg	4 h	Inhibit tachycardia	Decrease		Morris et al., 1995
Prodynorphyn	Rat	Striatum	S	10 nmol/day [10 nmol/day]	3 days		Decrease		Georgieva et al., 1995
TH	Rat	VTA	S	5 μg	4 days	Reduce operant behavior	Decrease		Skutella et al., 1994c
TPH	Mouse	i.c.v.	N	4 μg	4 days	Inhibit TPH activity			McCarthy et al., 1995
Vasopressin	Rat	i.c.v.	NS	10 to 100 μg [25 μg]	12 h	Induce licking	Decrease		Skutella et al., 1994b

AS, type of antisense oligodeoxynucleotide; Time, treatment time; NMDA, N-methyl-D-aspartate; ACh, acetylcholine; CCK, cholecystokinin; TRH, thyrotropin releasing hormone; GAD, glutamic acid decarboxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; CRH, corticotropin releasing hormone; S.N., substantia nigra; PAG, periaqueductal gray; HIP, hippocampus; HYP, hypothalamus; N.A., nucleus accumbens; VTA, ventral tegmental area; i.t., intrathecal; i.c.v., intracerebroventricular; PVN, paraventricular nucleus; S, phosphorothioate oligodeoxynucleotide; N, natural (unmodified) oligodeoxynucleotide.

Table 3. Effects of antisense expression vectors on the level and function of proteins in neuronal cells

Gene products	Cell type	Vector type	Stable or transient	Method of transfection	Function	Target protein	Target mRNA	Reference
Angiotensin AT1 receptor	Brainstem neuron	Retrovirus LNSV	Transient	Viral transduction	Inhibit c-fos and NE transporter mRNA			Lu and Raizada, 1995
Angiotensin AT1 receptor	Packaging PA317cells/ neuronal cells	Retrovirus LNSV	Stable and transient	Viral transduction mRNA	Inhibit c-fos	Decrease		Lu et al., 1995
CaM	PC12 cells	pMT	Stable	Lipofectin		Induce	Decrease	Davidkova et al., 1996
Dopamine D₂ receptor	Striatum	pCR3	Stable	Lipofectin	Inhibit dopamine release			Weiss et al., 1996d
GAP-43	PC12 cells	pMAM₀₀	Stable	Electroporation	Inhibit dopamine release	Decrease		Ivins et al., 1993
Gsα	Pituitary tumor GH3	pMThGH-111	Transient	Electroporation	Decrease cAMP	Decrease	Decrease	Paulssen et al., 1992
IGF-1	Neuroblastoma C6/B104 cells	Epstein-Barr virus	Stable	Lipofectin	Inhibit tumor growth	Decrease	Decrease	Trojan et al., 1992
n-myc	Neuroblastoma IMR-32 cells	pREP3	Stable	Electroporation	Inhibit neurite outgrowth	Decrease		Whitesell et al., 1991
Proenkephalin	NG 108-15 cells	pSVL SV 40	Stable	Calcium phosphate	Decrease apioid receptors	Decrease	Increase	Arany et al., 1992
Transglutaminase	Neuroblastoma SK-N-BE(2) cells	pSG5	Stable	Calcium phosphate	Decrease apoptosis			Melino et al., 1994

GAP, growth-associated protein; CaM, calmodulin; cAMP, adenosine 3',5'-cyclic monophosphate.

containing the translation initiation codon (AUG) (Rosolen et al., 1991; Amaratunga et al., 1993; Weiss et al., 1993). Other regions that have been targeted include the 5'-untranslated region, especially the sequence including the cap site, potential splice sites located at the intron–exon junctions, sequences within the different exons downstream of the initiation codon, and the 3'-untranslated regions (Laptev et al., 1994).

The mechanisms by which antisense oligodeoxynucleotides act depend on the targeted sequence: they can inhibit several steps in the processing of the primary RNA transcript (capping, methylation, poly A addition and splicing); they can inhibit the transport of the primary RNA transcript out of the nucleus; they can inhibit translation by hybridization arrest; and they can activate the metabolism of the RNA–DNA hybrids by the ubiquitous intracellular enzyme RNase H. The two major mechanisms of action of most antisense oligodeoxynucleotides, however, have been hybridization arrest and the activation of RNase H (Helene and Toulme, 1990).

Uptake and distribution of antisense oligodeoxynucleotides in brain

The use of antisense oligodeoxynucleotides in the central nervous system (CNS) depends upon their ability to penetrate into different brain regions, and on their ability to reach their specific targets in these regions. A number of laboratories have recently addressed the question of the distribution and metabolism of oligodeoxynucleotides in the brain, and have concluded that the distribution and half-life depend upon the size of the oligonucleotide, the chemical structure and the site of injection (Cossum et al., 1993; Agrawal et al., 1995; Crooke et al., 1996; Zhang et al., 1996). For example, Yee et al. (1994) compared the distribution of phosphorothioate and unmodified oligodeoxynucleotides in the brain. They showed that a digoxigenin-labelled, unmodified oligodeoxynucleotide antisense to the NMDA-R1 receptor mRNA was partly degraded within 4 h of injecting it into the lateral ventricle of the brain. Cells labelled with the NMDA-R1 oligonucleotide were detected in regions near the injection site, including the cortex, hippocampus and striatum. In another study (Sommer et al., 1993), the distribution of a FITC-labelled phosphorothioate oligodeoxynucleotide antisense to c-fos mRNA was determined after local administration into a discrete brain area, i.e. the corpus striatum. After infusion of the oligonucleotide into the striatum, it was distributed throughout large parts of the neostriatal area with a sharp concentration gradient. The corpus callosum appeared to

be a barrier for further diffusion. The uptake of the oligonucleotide into the cells appeared to be cell-type specific. Highest amounts were found in dopamine- and cyclic AMP-regulated, phosphoprotein-positive nerve cell bodies and some dendrites, but not axon terminals. To a much lesser extent the c-fos antisense oligomer was found in some glial cells.

Because we were interested in the use of antisense oligodeoxynucleotides targeted to the dopamine receptor mRNA, we determined the uptake and distribution in the brain of a phosphorothioate D_2 antisense oligonucleotide fluorescently labelled with fluorescein isothiocyanate (D_2 AS–FITC) (Zhang et al., 1996). When injected into the lateral ventricle of the mouse brain, a strong fluorescent signal, indicating the presence of labelled oligonucleotide, was evident in brain areas surrounding the ventricles, i.e. the corpus striatum, septum, hippocampus and hypothalamus. Fluorescent staining was also detected in the cerebral cortex, nucleus accumbens, substantia nigra and cerebellum.

Figure 1 shows an example of an experiment in which D_2 AS–FITC was injected intracerebroventricularly (i.c.v.) into mouse brain. As may be seen, a high fluorescent signal was detected in the corpus striatum and the septum of mouse brain after a single intraventricular injection (Fig. 1a). A greater signal was found in the ipsilateral injected side, although significant fluorescence was also seen in the contralateral side. Little fluorescent signal was seen in tissue consisting largely of white matter, such as the corpus callosum and anterior commissure. At a higher magnification, fluorescently labelled oligonucleotide can be seen, represented as punctuated fluorescent signals, which were localized to cell bodies as well as to proximal dendritic processes (Fig. 1b). These results indicate that after i.c.v. administration, the phosphorothioate-modified D_2 antisense oligonucleotide penetrates into several brain areas, including those regions with high levels of expression of D_2 dopamine receptors. Interestingly, not all cells appeared to be equally labelled, supporting the possibility that there might be a selective uptake and/or accumulation of oligodeoxynucleotides in certain cell types.

To estimate the stability of the phosphorothioate D_2 antisense oligodeoxynucleotide, it was labelled with digoxigenin (D_2 AS–DIG) and injected into the lateral ventricle of the mouse brain. The D_2 AS–DIG oligodeoxynucleotide remained intact in the mouse brain for approximately 1 day, was partly degraded 4 days after the injection, and appeared to be largely degraded after 16 days (Zhang et al., 1996).

Taken together, these results show that phos-

phorothioate-modified oligodeoxynucleotides rapidly penetrate into the brain from the cerebral ventricles and are rapidly taken up into certain, but not all, brain cells where they accumulate in cell bodies and proximal neurites; little accumulation is seen in the white matter. They remain unmetabolized for approximately 1 day, then become degraded. A major problem that remains before these compounds can enjoy widespread therapeutic use in neurobiological disorders is their ability to penetrate the brain from peripheral sites (Agrawal et al., 1995).

Overview of the application of antisense oligodeoxynucleotides in neurobiology

During the past few years, numerous examples of the application of antisense oligonucleotides in neurobiology have been widely studied, and several reviews of this field have already been published (Eng, 1993; McCarthy et al., 1993a; Chiasson et al., 1994; Harrison and Burnet, 1994; Pilowsky et al., 1994; Wahlestedt, 1994; Yung, 1994; Zon, 1995; Weiss et al., 1996a, 1997). The effects of antisense oligodeoxynucleotides have been examined in a variety of in vitro preparations of cells in culture (Table 1), as well as in several in vivo animal models (Table 2). Some of these studies are summarized briefly below.

Application of antisense oligodeoxynucleotides in vitro

It is impossible to summarize all the applications of antisense oligodeoxynucleotides, especially their use as tools to study theoretical problems in neurobiology. Therefore, we will give several examples illustrating the diversity of the studies which used antisense oligodeoxynucleotides as tools to inhibit the expression of proteins found in the nervous system (see Table 1). Antisense oligodeoxynucleotides targeted to the transcripts encoding non-receptor proteins, such as microtubule-associated proteins (Caceres and Kosik, 1990; Hanemaaijer and Ginzburg, 1991), oncogenes (Rosolen et al., 1991), immediate–early genes (Schlingensiepen et al., 1993;), synaptic proteins (Osen-Sand et al., 1993; Torre et al., 1994) and growth factors (Murphy et al., 1992), have been studied in vitro in continuous cell lines or cultured brain cells. They have proved invaluable as tools to study various aspects of neuronal proliferation and differentiation (Teichman-Weinberg et al., 1988; Shea et al., 1993), programmed neuronal cell death, and neuronal plasticity (Schlingensiepen et al., 1994). Oligodeoxynucleotides antisense to the mRNAs encoding distinct subtypes of G-proteins have been helpful in defining the signal transduction pathways activated by different neurotransmitters (Albert and Morris, 1994). In
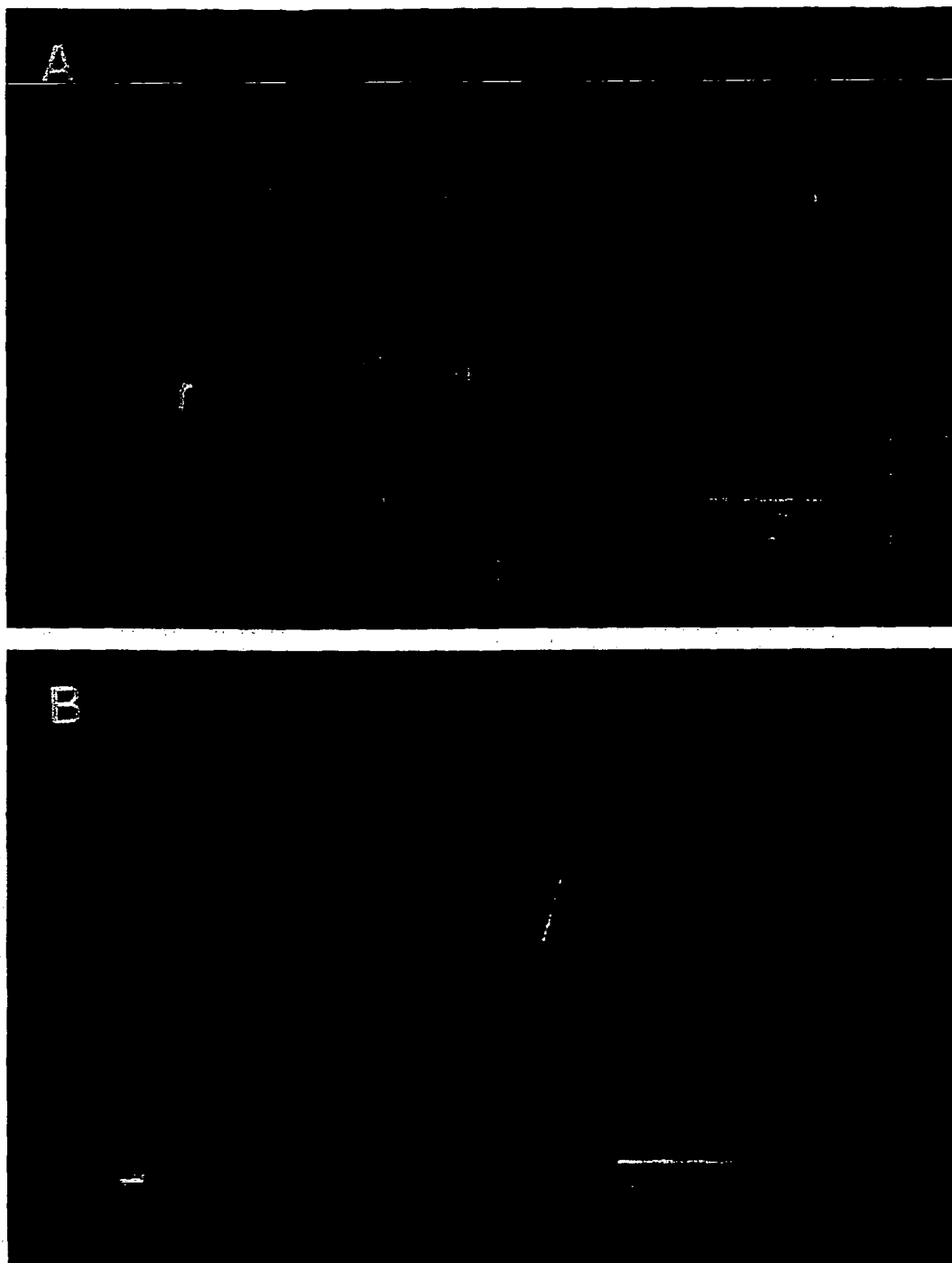
Fig. 1. Distribution of i.c.v. administered D_2 AS–FITC in the mouse brain. Mice were injected with D_2 AS–FITC (2 nmol/2 μl) i.c.v. once a day for 2 days. Twenty-four hours after the last injection, the mice were perfused through the left cardiac ventricle with 4% paraformaldehyde. The brains were removed, and serial coronal sections (16 μm) were cut on a cryostat. (A) ST, striatum; SP, septum; CC, corpus callosum; AC, anterior commissure. (B) Cells in corpus striatum with arrow pointing to a cell body and arrowhead pointing to a neuritic process (from Zhang *et al.*, 1996).

addition, an antisense strategy has been applied in neuroendocrinology to study the mechanisms of hormone action and secretion (Persaud and Jones, 1994).

An antisense oligodeoxynucleotide strategy has also been used *in vitro* to elucidate further the biology of tumor cells, and to develop more specific agents for treating abnormal cell growth in the nervous system. For example, antisense oligonucleotides have been used to down-regulate the proto-oncogene N-myc, which is amplified in tumors of neuroectodermal origin (Rosolen *et al.*, 1991). This treatment resulted in an increase in neuritic differentiation and an inhibition of the growth of neuroectodermal cells. Antisense oligodeoxynucleotides targeted to growth factors, such as basic fibroblast growth factor (bFGF), have been used to suppress the growth of melanoma and glioblastoma cells (Murphy *et al.*, 1992). The general conclusion that can be drawn from these and other studies is that the antisense approach, using both antisense oligodeoxynucleotides and antisense RNA generated by means of an expression vector, may hold promise for treating a variety of tumors of the nervous system. This approach will be discussed in more detail in a subsequent section, describing the application of an antisense RNA-producing vector, directed to the transcripts encoding the ubiquitous eukaryotic cell protein calmodulin.

An area in neurobiology that has benefited greatly from the application of antisense oligodeoxynucleotides as tools to inhibit the expression of specific proteins is the area of neurotransmitter receptor research. Many subtypes of neurotransmitter receptors have been identified recently in the brain, on the basis of molecular biological criteria, including several different subtypes of dopamine receptors (Civelli *et al.*, 1991), muscarinic acetylcholine receptors (Bonner *et al.*, 1987), and serotonin receptors (see Ruat *et al.*, 1993), but the functions of these newly uncovered receptors remain unknown.

The classical pharmacological means by which the function of receptors is uncovered is to use drugs that act as antagonists to the neurotransmitter receptor in question. These drugs, by binding to the respective receptors, inhibit the biochemical and behavioral events mediated by these receptors. Unfortunately, selective antagonists for the newly cloned neuroreceptor subtypes have not yet been developed, and many so-called "selective antagonists", upon further study, have proved to be rather non-selective in their actions. Antisense oligodeoxynucleotides, however, can be readily designed with the knowledge of the structure of the transcripts encoding the receptors or receptor subtypes, and can be employed to study the

functions of these receptors. Antisense oligodeoxynucleotides have already been used in *in vitro* studies of several receptors for neurotransmitters (Table 1), but most of the important information regarding the functions of the receptors has been derived from *in vivo* studies (Table 2). This is due to the fact that the results from cultured cells cannot always be directly extrapolated to animal models, and do not give information about the behavioral events mediated by the receptors. Furthermore, *in vivo* studies provide important information about the possibility of using antisense oligodeoxynucleotides, not only as specific tools to alter the expression of receptors for neurotransmitters, but also to treat diseases of the nervous system that may be associated with aberrant expression of certain neurotrammitter receptor subtypes.

APPLICATION OF ANTISENSE OLIGODEOXYNUCLEOTIDES *IN VIVO*

A large number of antisense oligodeoxynucleotides targeting neurobiologically important proteins have already been studied *in vivo* (Table 2). Some specific examples of these experiments are provided below.

Antisense oligodeoxynucleotides targeted to the mRNA transcripts encoding the dopamine receptor subtypes in the CNS

Dopamine is one of the major neurotransmitters in basal ganglia and mesolimbic areas of the brain, and is involved in the control of motor, emotional and endocrine functions (Maurer, 1981; Niznik, 1987; Riederer *et al.*, 1992; Salamone, 1992; Schaus *et al.*, 1993; Sage and Kark, 1994; Salamone, 1994). Hyperactivity of the dopaminergic system has been implicated in the positive symptoms of schizophrenia (Seeman, 1985; Carlsson, 1988), addiction to alcohol (Wise and Rompre, 1989) and cocaine (Bozarth, 1989), and in the development of tardive dyskinesia, often seen after long-term treatment with neuroleptic drugs (Seeman, 1985). The potential for using antisense oligodeoxynucleotides targeted to the dopamine receptor transcripts to study drug abuse (Weiss *et al.*, 1996a) and other dopamine-mediated behaviors (Weiss *et al.*, 1996b, 1997) has recently been reviewed.

The initially identified D_1 and D_2 subtypes of dopamine receptors (Spano *et al.*, 1978; Kebabian and Calne, 1979) have been more recently classified into five different subtypes on the basis of molecular biological data (Gingrich and Caron, 1993). The D_1-like dopamine receptor family includes the D_1 and D_5 subtypes, and the D_2-like family includes the D_2, D_3 and

D_4 subtypes. Some of the outstanding questions in this field are: why are there so many dopamine receptor subtypes, and what are their functions in the brain? In order to answer these questions it is necessary to use selective dopamine receptor agonists and antagonists. Unfortunately, like many other antagonists for neurotransmitter receptors, selective dopaminergic agents for the several dopamine receptor subtypes have not yet been developed. Furthermore, long-term treatment with the existing dopamine receptor antagonists often causes an up-regulation of the receptors they are designed to inhibit (O'Dell et al., 1990). This feedback mechanism may involve an increase in the expression of these receptors (Srivastava et al., 1990).

One means of selectively reducing the function of specific subtypes of dopamine receptors without changing the function of other neurotransmitter receptors is to use antisense oligodeoxynucleotides targeted to the transcripts encoding the various dopamine receptor subtypes. Furthermore, such a strategy may not lead to the up-regulation of the receptors commonly associated with classical dopamine receptor antagonists. Accordingly, several years ago we embarked on a study of the effects of antisense oligodeoxynucleotides as a novel approach to inhibit the expression of dopamine receptor subtypes in vivo. The effects of inhibiting the expression of specific subtypes of dopamine receptors on behaviors mediated by these receptors in several mouse models (6-hydroxydopamine-lesioned mice, mice treated with the irreversible D_2 antagonist fluphenazine-N-mustard (FNM) and normal mice), on specific biochemical (changes in the levels of dopamine receptors), and molecular (changes in the levels of dopamine receptor mRNAs) events were characterized.

D_1 dopamine receptor antisense oligodeoxynucleotides

The effect of a D_1 dopamine receptor antisense was evaluated in normal mice and in mice with unilateral lesions of the corpus striatum induced by the neurotoxin 6-hydroxydopamine, using certain behaviors that are characteristically produced by dopamine receptor agonists in these model systems. These behaviors include contralateral rotational behavior in response to challenge injections with D_1 and D_2 receptor agonists in 6-hydroxydopamine-lesioned mice, and grooming behavior and stereotypy in response to D_1 and D_2 dopamine receptor agonists, respectively, in normal mice.

A 20-mer phosphorothioate-modified oligodeoxynucleotide, antisense to a portion of the D_1 dopamine receptor mRNA, bridging the initiation codon (D_1 antisense) was administered i.c.v. to mice

(Zhang et al., 1994b). Treating normal mice with the D_1 antisense inhibited grooming behavior induced by the D_1 agonist SKF 38393, the reduction in grooming being related to the amount and length of time the antisense was given. Figure 2 shows that there was a significant decrease in D_1 agonist-induced grooming behavior in mice treated repeatedly with D_1 antisense when compared with that of mice treated with vehicle (artificial cerebrospinal fluid); the response returned to normal within 1 week after cessation of D_1 antisense treatment. The D_1 antisense also inhibited rotational behavior induced by SKF 38393 in 6-hydroxydopamine-lesioned mice, but not that induced by the D_2/D_3 receptor agonist quinpirole or by the muscarinic cholinergic agonist oxotremorine. Recovery from inhibition of rotational behaviors occurred after cessation of the D_1 antisense treatment (Zhang et al., 1994b).

D_2 dopamine receptor antisense oligodeoxynucleotides

Treatment of mice with a D_2 antisense also produced selective inhibition of D_2 dopamine-mediated behaviors. In these studies, a 20-mer phosphorothioate-modified oligodeoxynucleotide antisense to the area bridging the initiation codon of the mRNA encoding the D_2 dopamine receptor (D_2 antisense) was administered to mice with unilateral 6-hydroxydopamine lesions, and the ability of the antisense to inhibit specific D_2 receptor-mediated behaviors was evaluated (Weiss et al., 1993; Zhou et al., 1994). Administration (i.c.v.) of the D_2 antisense inhibited the rotational response to acute challenge injections with the D_2/D_3 agonist quinpirole, but did not block the contralateral rotations induced by either the D_1 agonist SKF 38393 or the muscarinic cholinergic receptor agonist oxotremorine. As observed in studies of the D_1 antisense, the inhibition was dependent upon the amount and length of time the D_2 antisense was given (Zhou et al., 1994). Significant reductions in D_2 dopamine-agonist-induced rotational behavior were seen within 1 day of repeated injections of the D_2 antisense, and almost complete inhibition was seen after 5 days of treatment (i.e. after 10 injections) (Fig. 3). Once again, recovery from inhibition occurred after cessation of the antisense treatment. Repeated treatment with the D_2 antisense reduced the density of D_2 dopamine receptors (as measured by D_2 receptor autoradiography) and the levels of D_2 receptor mRNA (as determined by in situ hybridization) in the lesioned striatum (Zhou et al., 1994).

Similar results to these were found when the D_2 antisense was administered directly into the corpus striatum of normal mice or into mice with unilateral
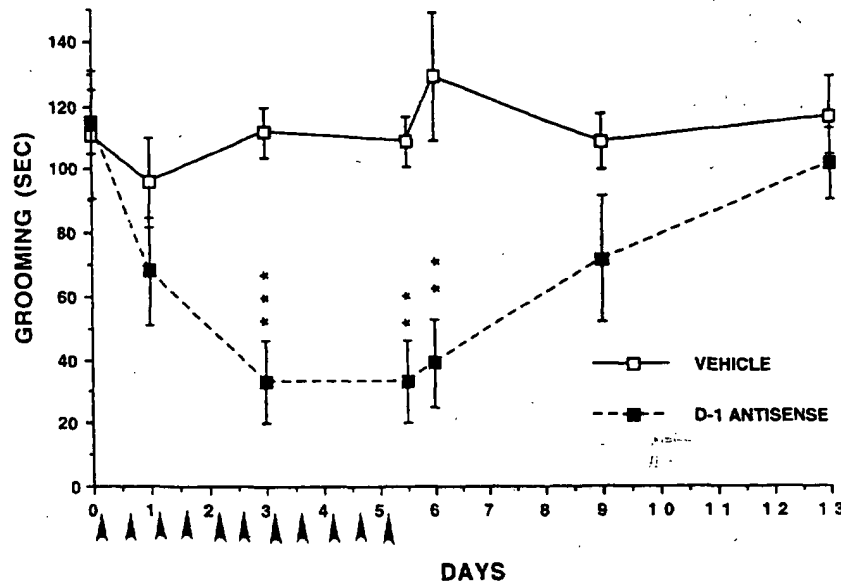
Fig. 2. Effect of D₁ antisense treatment on SKF 38393-induced grooming behavior. Mice were administered i.c.v. injections of vehicle and D₁ antisense at 12-h intervals (indicated by the arrowheads). Grooming behavior induced by SKF 38393 was measured 10 h after the second and sixth injection of vehicle or antisense, and at 0.5, 1, 4 and 8 days after the last injection. **$P < 0.01$ and ***$P < 0.001$ compared with vehicle treated mice (from Zhang et al., 1994b).

·6-hydroxydopamine lesions of the corpus striatum. Direct intrastriatal administration of the D₂ antisense in normal mice resulted in ipsilateral rotations in response to acute challenge injections with quinpirole.

This behavioral effect of the D₂ antisense can be explained by a decreased level of D₂ receptors in the side ipsilateral to the D₂ antisense injection, causing an imbalance of the D₂ receptors in the two striata.
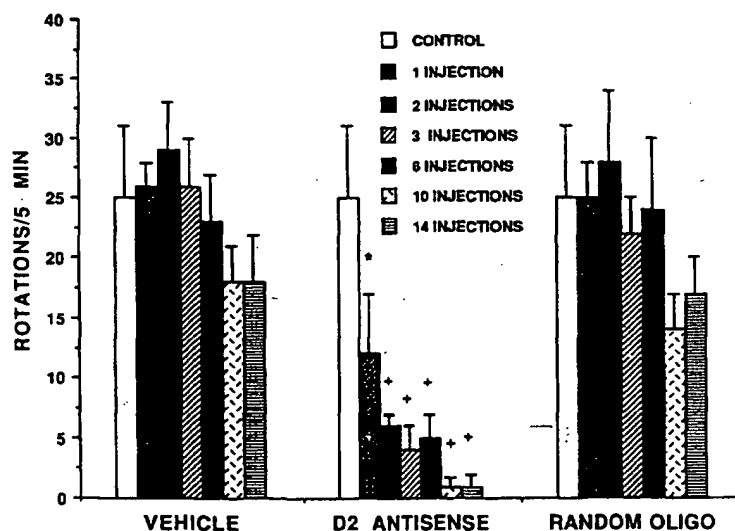

Fig. 3. Effect of D₂ antisense treatment on quinpirole-induced rotational behavior. Mice with unilateral 6-. hydroxydopamine-induced lesions received one to 14 i.c.v. injections of vehicle, D₂ antisense (2.5 nmol/2 μl) or random oligo (2.5 nmol/2 μl). The injections were made twice a day for 6 days and then once a day for 2 days. The rotational behavior induced by challenge injections of quinpirole was determined 10 h after each injection. *$P < 0.05$ compared with vehicle-treated mice. +$P < 0.001$ compared with vehicle- or random oligo-treated mice (from Zhou et al., 1994).

Injections of D_2 antisense into the striatum that was injected previously with 6-hydroxydopamine reversed the manifestations of dopaminergic supersensitivity seen upon challenge injections with quinpirole (Weiss *et al.*, 1994).

Other investigators have also carried out *in vivo* studies with oligodeoxynucleotides antisense to the D_2 dopamine receptor mRNA. In one such study a 19-mer D_2 antisense was infused i.c.v. into rats (Zhang and Creese, 1993). This treatment caused a down-regulation of D_2 receptors in the striatum and nucleus accumbens and induced specific changes in D_2-mediated behaviors (inhibited quinpirole-induced locomotor activation, elicited catalepsy and reduced spontaneous locomotor activity). In another study (Silvia *et al.*, 1994) a 17-mer D_2 antisense was delivered via an intracerebral cannula into the substantia nigra of rats. This treatment caused contralateral rotational behavior in response to subcutaneous injections of cocaine, and reduced the levels of D_2 dopamine receptors on the treated side. In striatal slices, the potency of the D_2 antagonist sulpiride in enhancing electrically stimulated dopamine release was significantly reduced on the antisense-treated side, consistent with a decrease in the population of striatal D_2 auto-receptors. These studies suggest that D_2 autoreceptors play a role in reducing the motor response elicited by cocaine (Silvia *et al.*, 1994).

D_2 dopamine receptor antisense oligodeoxynucleotides have also been used to inhibit the expression of D_2 receptors *in vitro* in cultured cells. In one study a D_2 antisense reduced by 57% the levels of D_2 receptors in WERI–27 retinoblastoma cells (Silvia *et al.*, 1994). Other investigators studied the effects of a D_2 antisense oligodeoxynucleotide in rat pituitary cells in order to understand more fully the functional contribution of D_2 receptors to the multiple transduction mechanisms involved in the actions of dopamine in these cells. After adding the antisense to the cells, the D_2 receptor agonist bromocriptine lost its ability both to inhibit adenyl cyclase activity and to reduce prolactin mRNA levels. In contrast, the inhibition of prolactin release induced by bromocriptine was affected minimally by the antisense treatment (Valerio *et al.*, 1994).

D_3 dopamine receptor antisense oligodeoxynucleotides

Relatively few studies on the application of D_3 dopamine receptor antisense oligodeoxynucleotides have been published so far. In one such study (Nissbrandt *et al.*, 1995), a D_3 antisense was continuously infused i.c.v. into the rat brain for 5 days. This treatment caused a decrease in the binding of

[^{3}H] spiperone in the limbic forebrain and an increase in dopamine synthesis in the nucleus accumbens. In studies from our laboratory we found that the i.c.v. administration of D_3 antisense in mice increased the locomotor activity induced by a continuous infusion of quinpirole (Weiss *et al.*, 1996a), further supporting a role for D_3 dopamine receptors in locomotor behavior (Svensson *et al.*, 1993).

Taken together, these studies indicate that antisense oligodeoxynucleotides targeted to the dopamine receptor mRNAs are useful for selectively blocking the functions of dopamine receptor subtypes, and that the antisense effect is completely reversible upon cessation of antisense treatment. They further suggest the possibility of using such antisense oligonucleotides therapeutically to treat conditions in which dopamine receptors play a role, and in reversing disorders associated with dopaminergic hyperactivity.

D_2 antisense oligodeoxynucleotides inhibit the synthesis of a functional pool of D_2 dopamine receptors

The fundamental question regarding the relationship between the levels of receptors and the effects that the receptors mediate arose again in experiments using D_2 antisense *in vivo*. These studies showed that D_2 antisense treatment markedly inhibited D_2 receptor-mediated behaviors, but produced only a relatively small reduction in the levels of D_2 dopamine receptors in the mouse striatum (Weiss *et al.*, 1993; Zhou *et al.*, 1994). A similarly poor correlation between the changes in the density of specific receptors and changes in behavioral events mediated by these receptors has also been observed after *in vivo* application of an antisense to the δ opioid receptor (Standifer *et al.*, 1994).

One possible explanation for this apparent discrepancy is that there may be more than one pool of receptors, and only a portion of the total pool is functionally active. According to this hypothesis, the newly synthesized pool of receptors constitutes the functionally active pool (Qin *et al.*, 1995). Accordingly, because antisense oligodeoxynucleotides inhibit the synthesis of receptor proteins, they would have a selective effect on this functional pool of receptors. As a result, antisense oligodeoxynucleotides would inhibit the functions mediated by receptors to a greater extent than they would inhibit the total pool of receptor proteins, the latter being determined from ligand binding assays which do not discriminate between functional and non-functional receptors.

To test this hypothesis and to explain this apparent dissociation between dopamine receptor-mediated behaviors and the levels of dopamine receptors, we

inhibited the total pool of D_2 dopamine receptors with the irreversible D_2 dopamine receptor antagonist FNM (Winkler et al., 1987), and then determined the effects of the D_2 antisense, administered i.c.v., on the rate of synthesis of D_2 receptors and on the recovery of D_2 receptor-mediated behaviors. FNM inactivates about 90% of the D_2 receptors within 4 h of treatment, after which the receptors return to normal levels. D_2 antisense treatment significantly inhibited the rate of recovery of D_2 receptors in the striatum of FNM-treated mice [Fig. 4(A, B)]. FNM treatment also produced a number of behavioral alterations, including catalepsy, and the inhibition of stereotypic behavior induced by the D_2/D_3 receptor agonist quinpirole. Both of these behaviors returned to normal within 4 days after FNM treatment; however, the D_2 antisense delayed the restoration of these FNM-induced behaviors. Figure 5 shows an example of one such experiment in which D_2 antisense treatment markedly reduced the recovery of normal stereotypic behavior induced by quinpirole in mice treated with FNM.

These studies demonstrated that the in vivo administration of the D_2 antisense decreased the rate of recovery of D_2 dopamine receptors and inhibited the recovery of D_2 receptor-mediated behaviors after irreversible receptor inactivation, and suggest that D_2 antisense treatment inhibits the synthesis of a functional pool of D_2 receptors.

Figure 6 shows a scheme depicting how the antisense may inhibit the synthesis of this functional pool of receptors. In this scheme there are several pools of receptors, some of which are intracellular and are therefore inaccessible for activation. They are nevertheless still measured by the used ligand binding assays. Evidence suggests that for receptors to be functional they must be membrane-bound and coupled to an appropriate G-protein (see Caron and Lefkowitz, 1993; Lefkowitz et al., 1993). We hypothesize that this relatively small, rapidly turning-over pool is the one that is reduced by antisense treatment. A similar mechanism of action of antisense oligodeoxynucleotides may apply to other such studies and should be taken into consideration in interpreting data derived from experiments exploring the in vivo application of antisense oligodeoxynucleotides targeted to the transcripts for neurotransmitter receptors, or for other biologically active proteins.

Effects of antisense oligodeoxynucleotides on the functions of other neuroreceptors and non-receptor proteins

An antisense oligodeoxynucleotide strategy has been used in vivo to study the functions of many other receptors and non-receptor proteins in the CNS (see Table 2). In these studies the antisense oligodeoxynucleotides have been injected into a variety of different brain regions, including the cerebral ventricles, corpus striatum, nucleus accumbens, substantia nigra, hippocampus and hypothalamus, depending on the specific brain protein to be targeted. These studies have yielded valuable information about the possibility of delivering antisense oligodeoxynucleotides to discrete regions in the brain in order to alter neurobiological events more selectively. Some examples are described below.

Antisense oligodeoxynucleotides targeted to the transcripts encoding other neurotransmitter receptors

Insofar as the use of antisense oligodeoxynucleotides targeted to the transcripts for other neurotransmitter receptors are concerned, Zhang et al. (1994a,b) reported that intraventricular infusion of an antisense oligodeoxynucleotide corresponding to a partial sequence of the mRNA encoding the M_1 muscarinic receptor reduced the density of muscarinic receptors in M_1 but not M_2 receptor-rich brain regions. The possible physiological functions of the 5-ht_6 receptor subtype were determined using an antisense oligodeoxynucleotide approach in rats (Bourson et al., 1995). Repeated i.c.v. treatment with this antisense gave rise to a specific behavioral syndrome of yawning, stretching and chewing. This finding, together with additional behavioral and biochemical data, suggested that 5-ht_6 receptors are functionally expressed in the rat brain, where one of their functions appears to be the control of cholinergic neurotransmission. An oligo-deoxynucleotide antisense to the N-methyl-D-aspartate (NMDA) receptor mRNA reduced the expression of the NMDA–R1 receptor subunit and reduced the volume of focal ischemic infarctions in the rat brain (Wahlestedt et al., 1993a).

Antisense oligodeoxynucleotides to peptide receptors

The transcripts encoding receptors for peptides in the CNS were among the early targets of antisense oligodeoxynucleotides in in vivo studies. In the study of Wahlestedt et al. (1993b) an oligodeoxynucleotide antisense to the neuropeptide Y–Y_1 receptors was administered to rats via the lateral ventricles. This treatment reduced the levels of neuropeptide Y_1 but not neuropeptide Y_2 receptors. Interestingly, these animals displayed behavioral signs of anxiety.
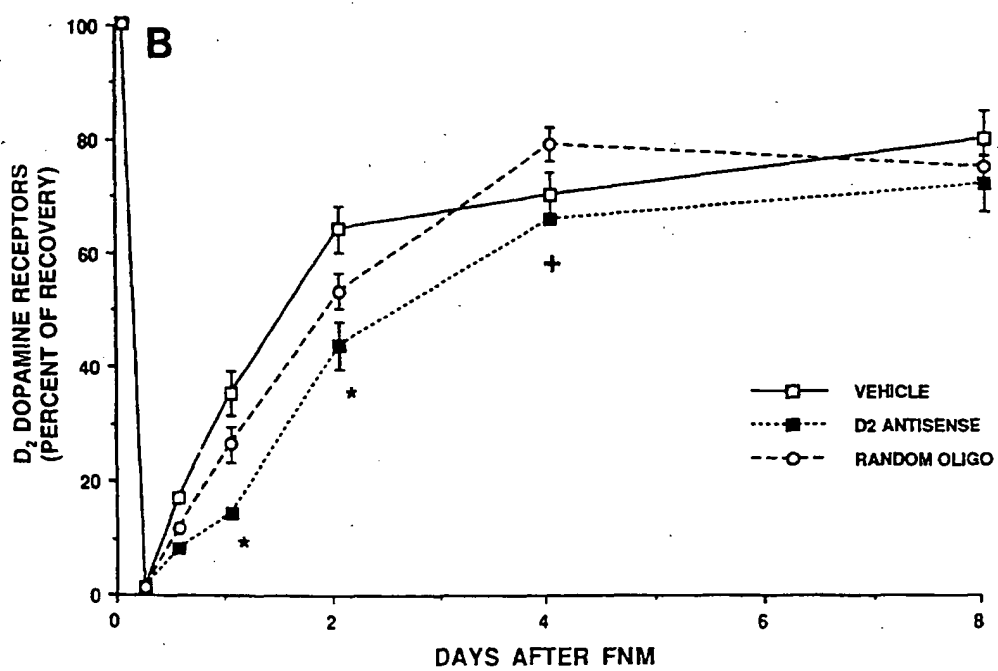
Antisense oligodeoxynucleotides to opioid receptors

An in vivo antisense oligodeoxynucleotide approach has also proved valuable to help understand the function of a number of opioid receptors, including the μ-

A

Vehicle

D_2 Antisense

Random Oligo

B

Figure B: D₂ DOPAMINE RECEPTORS (PERCENT OF RECOVERY) vs DAYS AFTER FNM
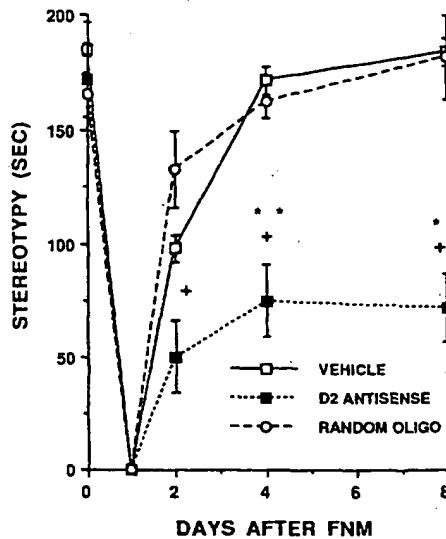
Legend:
- VEHICLE
- D2 ANTISENSE
- RANDOM OLIGO

Fig. 5. Effect of D_2 antisense on quinpirole-induced stereotypy in mice treated with FNM. Mice were administered D_2 antisense in FNM-treated animals as described in the legend to Fig. 4. At various times after the injection of FNM, the mice were administered challenge injections of quinpirole (5 μmol/kg, s.c.) and stereotypic behavior was measured. The maximum stereotypy score attainable was 300 s. In the absence of FNM treatment, vehicle-treated mice had stereotypy scores of 0. The mean values ± standard error are presented for five mice. Statistical analyses were performed with a two-way ANOVA followed by a Newman–Keuls test. $**P < 0.01$ compared with vehicle-treated mice; $^+P < 0.05$; $^{++}P < 0.01$ compared with values from random oligomer-treated mice. The results show that D_2 antisense treatment reduced the rate of recovery of quinpirole-induced stereotypy (from Qin et al., 1995).

(Rossi et al., 1994), δ- (Bilsky et al., 1994; Standifer et al., 1994; Tseng et al., 1994; Cha et al., 1995), and k- (Adams et al., 1994) opioid receptors. Repeated microinjection into the periaqueductal gray of an antisense oligodeoxynucleotide against the 5'-untranslated region of the μ-opioid receptor-clone 1

completely blocked the analgesic actions of morphine (Rossi et al., 1994). Furthermore, the pharmacological profiles of the δ-opioid receptor subtypes have been characterized by administering antisense oligodeoxynucleotides intrathecally or i.c.v. into rats, and determining the inhibitory effects of the oligodeoxynucleotides on the antinociceptive actions of putative subtype-selective δ-agonists and selective μ- and k-agonists. The intrathecal administration of an antisense to the δ-opioid receptor thus inhibited the antinociceptive actions of δ_1-agonists but not that of μ-agonists (Standifer et al., 1994). In a similar study, the i.c.v. administration of an δ_1-opioid receptor antisense oligodeoxynucleotide selectively inhibited the antinociceptive actions of a δ-agonist, but not that induced by a selective k-agonist (Bilsky et al., 1994). Finally, i.c.v. treatment with an antisense oligodeoxynucleotide to the k-opioid receptor selectively inhibited the analgesic effect of the k-opioid agonist spiradoline, but not that of μ- or δ-opioid agonists (Adams et al., 1994). These studies provided compelling evidence for the specificity of action of antisense oligodeoxynucleotides targeted to the transcripts encoding specific opioid receptor subtypes.

Antisense oligodeoxynucleotides to steroid receptors

In an interesting series of studies, McCarthy et al. (1993b, 1994a) reported on the use of antisense oligodeoxynucleotides to investigate the role of oxytocin receptors, estrogens and progesterone in reproductive function. In one such study, the functions of the oxytocin receptor in the ventromedial hypothalamus were investigated by injecting an oligodeoxynucleotide antisense to the 5'-region of the human oxytocin receptor mRNA into the ventromedial hypothalamus of hormonally primed rats. Infusion of the antisense oligomer significantly reduced the frequency and intensity of lordosis in female rats primed with estro-

Fig. 4. Effect of D_2 antisense on the recovery of D_2 dopamine receptors in striatum of mice treated with fluphenazine-N-mustard (FNM). Mice were pretreated with a single injection of the selective irreversibly acting D_2 dopamine receptor antagonist FNM (20 μmol/kg i.p.). Starting 4 h later, they were administered intraventricular injections of vehicle (2 μl of artificial cerebrospinal fluid), D_2 antisense (2.5 nmol/2 μl), or random oligodeoxynucleotide (2.5 nmol/2 μl) twice a day for 8 days. The brains were removed at various times after treatment with FNM, always 2 h after the last injection of vehicle or oligomer. Then, 12-μm coronal sections were incubated with [^{3}H]-raclopride (2 nM), and the slides were processed for receptor autoradiography. (A) An example of the receptor autoradiographic analysis of D_2 dopamine receptors in the brains of mice 2 days after treatment with FNM. (B) A quantitative analysis of the receptor density in mouse striatum. Optical densities of the dorsolateral areas of the striatum were analysed with a DUMAS image analyser. Points, mean values from five mice; error bars, standard error. Statistical comparisons between antisense-treated mice with either vehicle-treated or random oligomer-treated mice were carried out with a two-way analysis of variance (ANOVA) followed by a Newman–Keuls test. $*P < 0.05$ compared with vehicle- or random oligomer-treated mice; $^+P < 0.05$ compared with random oligomer-treated mice (from Qin et al., 1995).
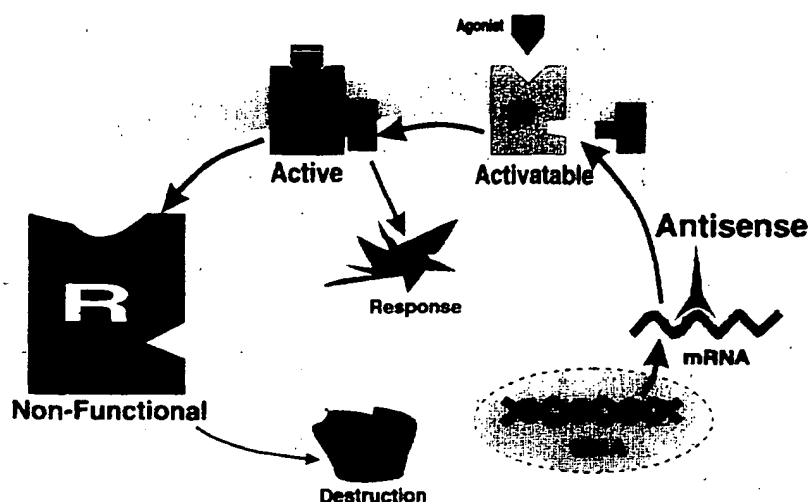
LIFE CYCLE OF A RECEPTOR

Fig. 6. Life cycle of a receptor. The figure depicts the scheme whereby the newly synthesized receptors translated from the mRNA are coupled to G-proteins and become active upon interaction with the agonist; the active form of the receptor then produces its characteristic response. The receptors then invaginate into the interior of the cell becoming non-functional and are eventually destroyed. All forms of the receptor (activatable, active and non-functional) are detected by the usual receptor ligand binding assay. We hypothesize that an antisense oligodeoxynucleotide, by blocking the synthesis of the activatable pool of receptors, greatly reduces the function of the receptors without producing a proportional change in the total levels of receptors, as measured by receptor ligand binding assays. Heavy and light arrows suggest relatively rapid or slow reaction rates, respectively. The relative size of the different pools of receptors is suggested by the size in the diagram. The scheme attempts to explain how a comparatively small change in the total number of receptors can produce marked changes in their function (from Weiss et al., 1997).

gen. It also significantly increased the number of rejection behaviors exhibited by estrogen-primed females. In contrast, when females were injected with estrogen and progesterone to increase their sexual receptivity, the antisense oligomer infusion had no effect on sexual behavior. These behaviors were accompanied by a 31% reduction of oxytocin receptor binding in the ventromedial hypothalamus.

Antisense oligodeoxynucleotides to immediate–early genes

An antisense strategy has been used to examine the role of immediate–early genes in specific sites in the brain in vivo. Several studies have demonstrated that decreasing the expression of c-fos by direct infusion of antisense oligonucleotides into discrete brain regions produces behavioral changes (Chiasson et al., 1992; Dragunow et al., 1993; Heilig et al., 1993; Sommer et al., 1993; Hooper et al., 1994). In an early study, an antisense oligodeoxynucleotide directed to c-fos mRNA was injected into the corpus striatum of rats via an implanted cannula. This treatment reduced amphetamine-induced production of c-fos (Chiasson et al., 1992). In a more recent report, unilateral

infusion into the rat neostriatum of an antisense oligodeoxynucleotide to c-fos mRNA led to the rapid induction of ipsilateral rotational behavior after d-amphetamine administration, and eliminated the in vivo expression of fos-like immunoreactivity within the infused regions of the neostriatum (Sommer et al., 1993). Similarly, the intrastriatal injection of rats with an antisense to c-fos caused ipsilateral rotations in response to parenteral injections of amphetamine or apomorphine (Dragunow et al., 1993). The antisense oligonucleotide also strongly inhibited the amphetamine-induced expression of c-fos and jun-b in striatal neurons. The bilateral administration of an antisense oligodeoxynucleotide to c-fos into the nucleus accumbens of rats blocked cocaine-induced locomotor stimulation, thus suggesting a role played by c-fos in mediating the psychostimulant effects of cocaine (Heilig et al., 1993). Furthermore, an antisense oligodeoxynucleotide to c-fos, when superfused into the rat spinal cord, was found to suppress the expression of heat-induced c-fos without affecting other members of the fos and jun family (Gillardon et al., 1994). This latter study has provided a technique to determine the functions of immediate–early genes in sensory spinal

cord neurons. Finally, antisense oligonucleotides to the immediate–early genes jun-b and c-fos have been used to study the light-induced phase shifts of the circadian rhythm in rats (Schlingensiepen et al., 1994). Injection of these antisense oligodeoxynucleotides into the third ventricle reduced the levels of the two transcription factors in vivo and prevented the light-induced phase shifts of the circadian rhythm.

Antisense oligodeoxynucleotides to non-receptor proteins

Among the several non-receptor proteins which have been studied thus far are hormones, angiotensinogen (Wielbo et al., 1995) and vasopressin (Skutella et al., 1994b); enzymes, glutamic acid decarboxylase (GAD) (McCarthy et al., 1994b) and tyrosine hydroxylase (Skutella et al., 1994c); and endorphins (Georgieva et al., 1995). For example, the administration of oligodeoxynucleotides antisense to the mRNAs encoding the g-aminobutyric acid (GABA) synthesizing enzymes GAD_{65} and GAD_{67}, into different brain regions of ovariectomized rats that had been chronically treated with estrogen, showed that these enzymes modulate female reproductive behavior (McCarthy et al., 1994b).

Clearly, even from this very brief review, one can conclude justifiably that antisense oligodeoxynucleotides targeted to transcripts encoding proteins in the nervous system hold great promise for studying the biological activity of these proteins, and for selectively altering their function.

Specificity of antisense oligodeoxynucleotides

A major advantage of the antisense approach over traditional pharmacological methods to alter biological activity is that it allows the design of agents that should have a high degree of specificity. Moreover, these agents are relatively easily and rapidly designed and are readily synthesized. However, in order to draw conclusions from the results with antisense oligomers, it is necessary to include several controls to ensure that the observed effects are indeed specific. This stricture gains greater importance because of recent studies suggesting that some antisense oligodeoxynucleotides have "specific" non-antisense effects (Stein and Cheng, 1993; Hertl et al., 1995; Vaerman et al., 1995).

First, it is important to select an oligodeoxynucleotide of an appropriate length and with a sequence that is highly specific for the intended target. This oligodeoxynucleotide should not hybridize to other nucleotide sequences in the cell or to any biologically active proteins. Because the shortest

nucleotide sequence that is likely to be unique within the mRNA pool is 13 nucleotides in length (Woolf et al., 1992), it has been argued that antisense oligodeoxynucleotides should be at least 13-mer. Most of the studies, therefore, have been carried out with oligonucleotides between 15 and 20 nucleotides in length. Oligonucleotides of larger size, although less likely to hybridize to non-intended target sequences, would have a poorer penetration into the cell, and would require greater costs for synthesis.

Computer programs have proved to be an indispensable aid in the appropriate design of oligodeoxynucleotides (we have found it most convenient to use the Genepro software or the Find Patterns from the GCG package). These programs perform nucleic acid sequence analyses and searches for similarity between a given oligonucleotide and the other nucleic acid sequences that have been included in the database. In the design of antisense oligodeoxynucleotides we have chosen a phosphorothioate-modified sequence which is at least 20% different from all non-target cDNA sequences currently deposited in the database.

Even though the antisense oligodeoxynucleotides are designed after stringent selection to find a unique sequence, multiple controls should be included to confirm the specificity of the effect. One such control is to include a "random" or "missense" oligomer, i.e. an oligonucleotide with the same base composition as the antisense sequence but in a scrambled order. Although "sense" oligodeoxynucleotides have frequently been used as negative controls, the use of a sense control is not recommended, because it may form a triplex with DNA or bind to proteins needed for the expression of the target genes. For example, in the studies with the D_1 and D_2 dopamine receptor antisense oligodeoxynucleotides, we compared the effects of the antisense oligomers with those of a random control on specific behaviors, levels of dopamine receptors and dopamine receptor mRNAs. The D_1 and D_2 random oligonucleotides did not cause specific changes in any of the parameters measured (Weiss et al., 1993; Zhang et al., 1994b; Zhou et al., 1994).

The specificity of the effect of the antisense oligodeoxynucleotides should also be demonstrated at the level of the specific protein which is being targeted. For example, repeated or continuous i.c.v. administration of a D_2 antisense reduced the levels of D_2 dopamine receptors in the corpus striatum, but did not cause any changes in the levels of the D_1 dopamine receptors (Weiss et al., 1993; Zhang and Creese, 1993; Zhou et al., 1994). It should be noted that in some cases small to modest changes in the levels of the target

mRNA are also seen (Zhou et al., 1994; Landgraf et al., 1995). However, changes in the levels of the specific targeted mRNA are not always observed after antisense treatment, the reason being that in order to achieve a reduction in the levels of the mRNA, the mRNA:oligodeoxynucleotide hybrid must be a substrate for RNase H.

Another approach to confirm the specificity of the antisense in in vivo studies of receptors for neurotransmitters is to determine the specificity of the behavioral effects. For example, an antisense to the D_2 dopamine receptor inhibited stereotypic behavior induced by the D_2/D_3 agonist quinpirole in mice, but did not inhibit grooming behavior induced by the D_1 agonist SKF 38393. In contrast, the D_1 antisense inhibited grooming behavior induced by SKF 38393, but did not affect stereotypic behavior induced by quinpirole (Fig. 7). A further indication for a specific and reversible inhibition of the individual dopamine receptor subtypes with antisense oligonucleotides was provided in studies showing that after the cessation of D_1 antisense treatment, the grooming behavior in response to SKF 38393 returned to that of control values (Zhang et al., 1994b).

Problems associated with the therapeutic application of antisense oligodeoxynucleotides

Antisense oligodeoxynucleotides have proved to be invaluable tools in neurobiology, but there are several problems which have to be overcome before they can be applied as therapeutic agents. A major problem is the requirement for the multiple administration of these compounds in order to achieve the antisense effect. This can be a particularly serious obstacle when the oligodeoxynucleotides have to be administered for therapeutic purposes to treat disorders of the CNS. Another problem is their poor penetration into cells because of their large size. This poor penetration into cells, however, may be more pronounced in in vitro preparations than in in vivo experiments. Several studies in vivo show remarkably strong antisense effects (Zhang et al., 1994b; Zhou et al., 1994; Bourson et al., 1995; Landgraf et al., 1995; Qin et al., 1995). Perhaps cells grown in vitro have an altered character or an altered environment compared with cells in vivo, and these changes might account for the differences in the relative ability of oligodeoxynucleotides to penetrate cells. The poor penetration of antisense oligodeoxynucleotides into the CNS is certainly a problem when these agents are administered peripherally, as there is very poor penetration of the oligodeoxynucleotides into the brain when injected parenterally (Agrawal et al., 1995). However, oligo-

deoxynucleotides readily penetrate into the brain when injected directly into the cerebroventricular fluid (Yee et al., 1994; Zhang et al., 1996; see also Fig. 1).

Sometimes the large doses of oligodeoxynucleotides required to achieve an antisense effect may cause nonspecific toxicity to the cells or to the experimental animals. This has been seen mainly with phosphorothioate oligodeoxynucleotides, which may bind non-specifically to proteins (Perez et al., 1994). For example, phosphorothioate antisense oligodeoxynucleotides to c-fos have shown neurotoxic damage after repeated daily infusions into the amygdala of rats (Chiasson et al., 1994). Increasing the interval between infusions, however, drastically reduced the damage to the brain parenchyma. Non-specific toxicity of phosphorothioate oligodeoxynucleotides was observed in studies from our laboratory, where a number of behavioral abnormalities were seen after the i.c.v. administration of D_2 antisense into mice (Weiss et al., 1993; Zhou et al., 1994). These abnormal behaviors, however, were short-lasting and became progressively less with further treatment with the antisense.

In addition, some of these non-specific effects may be obviated somewhat by taking into account the relatively sharp concentration–response curves that antisense compounds possess. Traditional antagonists often have concentration–response curves whose minimum and maximum concentrations extend over several orders of magnitude. However, it is our experience that antisense oligodeoxynucleotides have a much sharper concentration–response curve, usually having specific effects over only approximately a five-fold range of concentrations (i.e. less than a single order of magnitude). If the investigator is unaware of this, many experiments may show either no effect (too low a concentration) or a non-selective effect (too high a concentration) of the compounds.

Finally, at present the cost of synthesizing oligodeoxynucleotides is relatively high and may limit their application for therapeutic purposes. However, newer and cheaper methods for synthesizing these agents are currently being developed (Burger, 1993).

Advantages of antisense oligodeoxynucleotides over traditional pharmacological antagonists

Even though problems exist with the application of antisense oligodeoxynucleotides, they have several advantages over traditional pharmacological therapies. A major advantage of antisense oligodeoxynucleotides is their high specificity. An antisense oligodeoxynucleotide can be specific not only for a certain receptor type, but also for a specific subtype
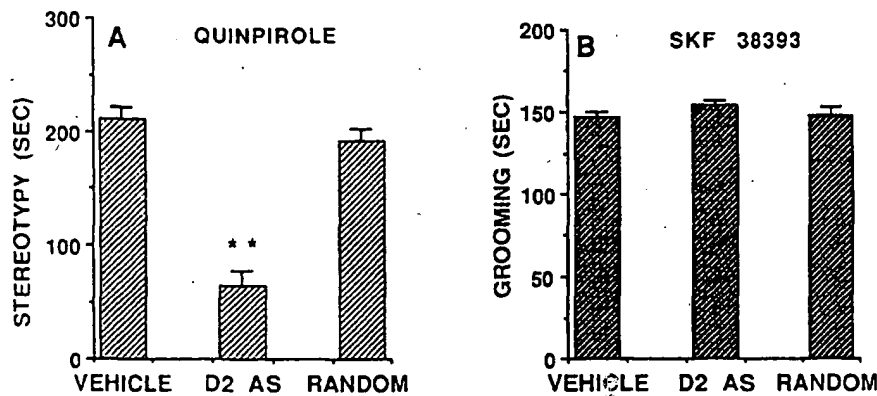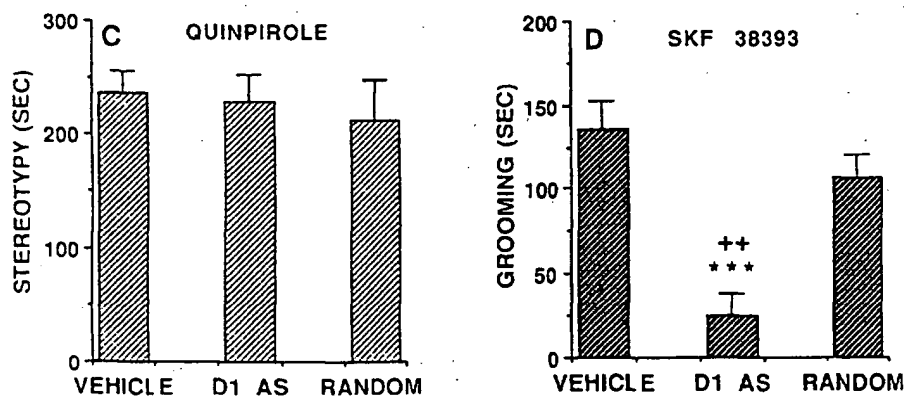
D2 ANTISENSE

D1 ANTISENSE

Fig. 7. Effect of intraventricular administration of D_2 antisense and D1 antisense on behaviors induced by quinpirole and SKF 38393 in mice. Mice were administered intraventricular injections of vehicle ($2\,\mu l$ of artificial cerebrospinal fluid), a random oligodeoxynucleotide ($2.5\,nmol/2\,\mu l$), and either a D_2 antisense ($2.5\,nmol/2\,\mu l$) or a D_1 antisense ($2.5\,nmol/2\,\mu l$) oligodeoxynucleotide twice a day for 6 days. Behaviors induced by challenge injections of the D_2 agonist quinpirole and the D_1 agonist SKF 38393 were measured 10 h after the last injection of vehicle or oligomers. (A) and (C) represent the stereotyped behavior induced by acute injections of quinpirole ($20\,\mu mol/kg$, s.c.) in D_2 antisense- and D_1 antisense-treated mice, respectively. (B) and (D) represent the grooming behavior induced by acute injections of SKF38393 ($80\,\mu mol/kg$, s.c.) in D_2 antisense- and D_1 antisense-treated mice, respectively. Each column represents the mean value from four to five mice. Vertical brackets indicate the standard error. $**P < 0.01$ compared with the corresponding values from vehicle-treated or random oligodeoxynucleotide-treated mice. $***P < 0.001$ compared with the values from vehicle-treated mice. $++P < 0.01$ compared with the values from random oligodeoxynucleotide-treated mice. The results show that treatment with D_2 antisense and D_1 antisense caused selective blockade of D_2- and D_1-mediated behaviors, respectively (from Weiss et al., 1997).

of receptor. For example, it has been possible to design antisense oligodeoxynucleotides which selectively inhibit the D_1 or D_2 dopamine receptor subtypes (Zhang et al., 1994b; Zhou et al., 1994). In contrast, most of the conventional dopamine receptor antagonists are relatively non-selective, because they not only interact with more than one dopamine receptor subtype (Pugh et al., 1985; Malmberg et al., 1993),

but they also inhibit several other neurotransmitter receptors, including cholinergic, adrenergic, serotonergic and histaminergic receptors (Peroutka and Snyder, 1980). Furthermore, the continuous administration of dopamine receptor antagonists can induce long-term and sometimes irreversible up-regulation of dopamine receptors, changes which are thought to be responsible for the development of the motor side effects (tardive dyskinesia) seen after their chronic clinical use (Creese and Snyder, 1980; Ebadi and Hama, 1988). The application of D_1 and D_2 receptor antisense oligomers has shown that their effects are completely reversible upon cessation of antisense treatment (Zhang *et al.*, 1994b; Zhou *et al.*, 1994). Other potential advantages of using antisense oligodeoxynucleotides are that more is known about the structure of RNA than about the protein targets, and more is known about the interactions between nucleotides than between drugs and proteins. Finally, as has already been mentioned, antisense oligodeoxynucleotides can easily be designed and readily and rapidly synthesized. However, as also mentioned earlier, caution must be exercised in interpreting the results of experiments with antisense compounds as non-antisense effects of antisense oligodeoxynucleotides have been reported (Stein and Cheng, 1993; Hertl *et al.*, 1995; Vaerman *et al.*, 1995).

ANTISENSE RNA EXPRESSION VECTORS

An alternative approach to the use of antisense oligodeoxynucleotides to inhibit the expression of specific proteins is to generate antisense RNA inside the cell. This can be accomplished by administering a vector containing a DNA sequence encoding an antisense RNA. The vectors which have been used thus far to produce antisense RNA have either been eukaryotic expression plasmids or viral vectors.

Advantages of antisense RNA expression vectors over antisense oligodeoxynucleotides

A large body of evidence suggests that transgenes encoding sense strands of mRNA can be delivered to the CNS with the use of suitable vectors (see Ridet and Privat, 1995). A similar technique may be used to deliver DNA sequences encoding antisense RNA molecules. There are several potential advantages of using an expression vector to generate antisense RNA intracellularly. The antisense RNA may be delivered into the CNS with a greater efficiency than antisense oligodeoxynucleotides, because vectors may be designed to penetrate more readily into the nervous system. The duration of action may be far longer with antisense RNA expression vectors than with antisense oligodeoxynucleotides, because the vector may continually express the antisense inside the cell over long periods of time. If the vector is incorporated into the genome, the effects would be expected to be quite long (several months), but even if the vector only remains episomal, it may still be expressed for several weeks. This contrasts with a half-life for antisense oligodeoxynucleotides of only a few days at most (Zhang *et al.*, 1994b, 1996; Zhou *et al.*, 1994). The vector may be targeted to specific cell types by taking advantage of cell-specific protein antigens that exist on certain cells. By coating the vectors with appropriate antibodies, the vector may be taken up selectively into those cells having the specific antigen. Finally, the vector may be designed to be expressed only in certain cells even if it penetrates into several cell types. This can be achieved if tissue-specific promoters are incorporated into the vector, and if unique transcription activating factors are present in the target cell. If these promoters are inducible, it might even be possible to regulate the production of the antisense RNA inside the cell by administering the transcription activators.

There are, however, several potential problems associated with the delivery and expression of antisense RNA into neurons by means of expression vectors. In studies *in vitro* on stably transfected cultured cells, non-specific effects could be observed because of positional effects resulting from the integration of the vector into the genome. Among the most efficient vectors for transfecting neurons are herpes simplex viral vectors which, unfortunately, very often possess neurotoxicity (Pakzaban *et al.*, 1994). Adenoviruses are also suitable for transfecting neurons, but they frequently elicit an immune response leading to a decrease in the level of expression of the recombinant sequence (Neve, 1993). Biologically, non-viral plasmid vectors would be expected to have relatively few undesirable effects for *in vivo* gene transfer, but the efficiency with which these vectors penetrate into neurons both *in vitro* and *in vivo* is relatively low. One approach is to increase their uptake through the use of cationic lipids, which can give very high transfection efficiencies *in vitro* (Felgner *et al.*, 1987, 1994) and which more recently have been used to transfect neurons *in vivo* (Roessler and Davidson, 1994) and *in vitro* (Davidkova *et al.*, 1996).

Use of antisense RNA expression vectors

Several examples of the *in vitro* application of the use of antisense RNA expression vectors to block the expression of specific proteins found in neuronal cells

(GAP–43, proenkephalin, calmodulin), glial cells (GFAP) and tumors of the nervous system (n-myc, IGF-1) are shown in Table 3. In these studies, the antisense RNA vector approach has been used to elucidate the role of specific proteins in the development and physiology of nerve cells. For example, herpes simplex virus type 1 vector, producing GAP–43 antisense RNA has been stably transfected into PC12 cells in order to analyse the interaction of GAP–43 with the signal transduction systems during neurotransmitter release (Ivins et al., 1993).

Most of the studies published so far involve the introduction of antisense RNA vectors into continuous cell lines that originate from tumors of the nervous system (neuroblastoma, glioma cells, PC12 cells). This is because primary neuronal cultures are difficult to transfect because they do not normally divide and have a limited life in culture. To improve the cellular uptake of expression plasmids, they have been complexed with cationic lipid preparations. As with the antisense oligodeoxynucleotide experiments, it is necessary to select highly specific antisense sequences and to use appropriate controls (an empty cloning vector) to assess non-specific effects. An example of the use of an antisense RNA expression vector in PC12 pheochromocytoma cells is shown below.

Inhibition of calmodulin with antisense RNA produced by a eukaryotic expression plasmid

The ubiquitous eukaryotic cell protein calmodulin is involved in many neuronal processes, including Ca^{2+}-dependent regulation of cyclic nucleotide metabolism, Ca^{2+} transport, protein phosphorylation-dephosphorylation cascades, ion transport, cytoskeletal function and cell proliferation (Thermos and Weiss, 1985; Means, 1994). It is also expressed at higher than normal levels in many malignancies such as glioblastomas, mammary carcinomas and leukemic cells (Hait et al., 1985; Lazo et al., 1986; Hardcastle et al., 1995; Takemoto and Jilka, 1995). An important role for calmodulin in biology is supported by the facts that calmodulin is widely distributed in all eukaryotic cell types, has a highly conserved protein sequence, and is represented by multiple genes in rodents and humans, all of which encode a protein with the same sequence.

That calmodulin plays a particularly important role in neurobiological events is evidenced by the fact that the protein (Zhou et al., 1985) and its transcript (Cimino et al., 1990; Roberts-Lewis et al., 1990) are found in extremely high concentrations in the CNS, it is localized to postsynaptic densities (DeLorenzo, 1981), and is involved in several important enzymatic

reactions that occur in the nervous system, including the activity of tyrosine hydroxylase (Griffith and Schulman, 1988), and the synthesis and metabolism of cyclic AMP (Weiss and Wallace, 1980). In order to elucidate the role of calmodulin in neuronal proliferation and differentiation, we stably transfected PC12 cells with a eukaryotic expression vector containing the gene for calmodulin gene I in the sense and antisense orientation relative to the metallothionein promoter of a eukaryotic expression plasmid vector (Davidkova et al., 1996). PC12 cells in which the levels of calmodulin were increased showed an increased rate of proliferation (Fig. 8C), whereas cells in which calmodulin levels were reduced by expressing the calmodulin antisense RNA (Fig. 8D) had significantly slower rates of growth than did parental, untransfected cells (Fig. 8A) or PC12 cells stably transfected with the empty expression vector (Fig. 8B). In addition to the slower growth, the stable calmodulin antisense transfectants exhibited the spontaneous outgrowth of long neuritic-like processes (Fig. 8D). These results indicate that by reducing the levels of calmodulin in PC12 cells, it is possible to inhibit cell proliferation and to induce terminal differentiation. Accordingly, they suggest that the use of an antisense RNA produced by an expression vector may provide a possible approach to limit the growth and induce differentiation of tumors of the nervous system.

This general strategy may also be used to produce long-term inhibition of the effects mediated by other neuronal proteins. Indeed, we have recently shown that a single intracerebral injection of a plasmid vector expressing a D_2 antisense RNA produced a specific inhibition of D_2 dopamine receptor-mediated behaviors in mice, and that this inhibition lasted for several weeks (Weiss et al., 1996c).

SUMMARY AND CONCLUSIONS

Antisense oligodeoxynucleotides have been used to inhibit the expression of specific proteins in the nervous system both in vitro and in vivo. They penetrate into many brain regions and are taken up into brain cells after i.c.v. administration, suggesting that they can reach their target RNA. In neurobiology, antisense oligodeoxynucleotides have been particularly useful for studying in vivo the role of several neuroreceptors and neuromodulators, including various newly uncovered subtypes of receptors for neurotransmitters. Using antisense oligodeoxynucleotides to the D_1 and D_2 dopamine receptors in vivo in mice, it has been possible to inhibit the expression of these receptors specifically, and to modify specific behaviors
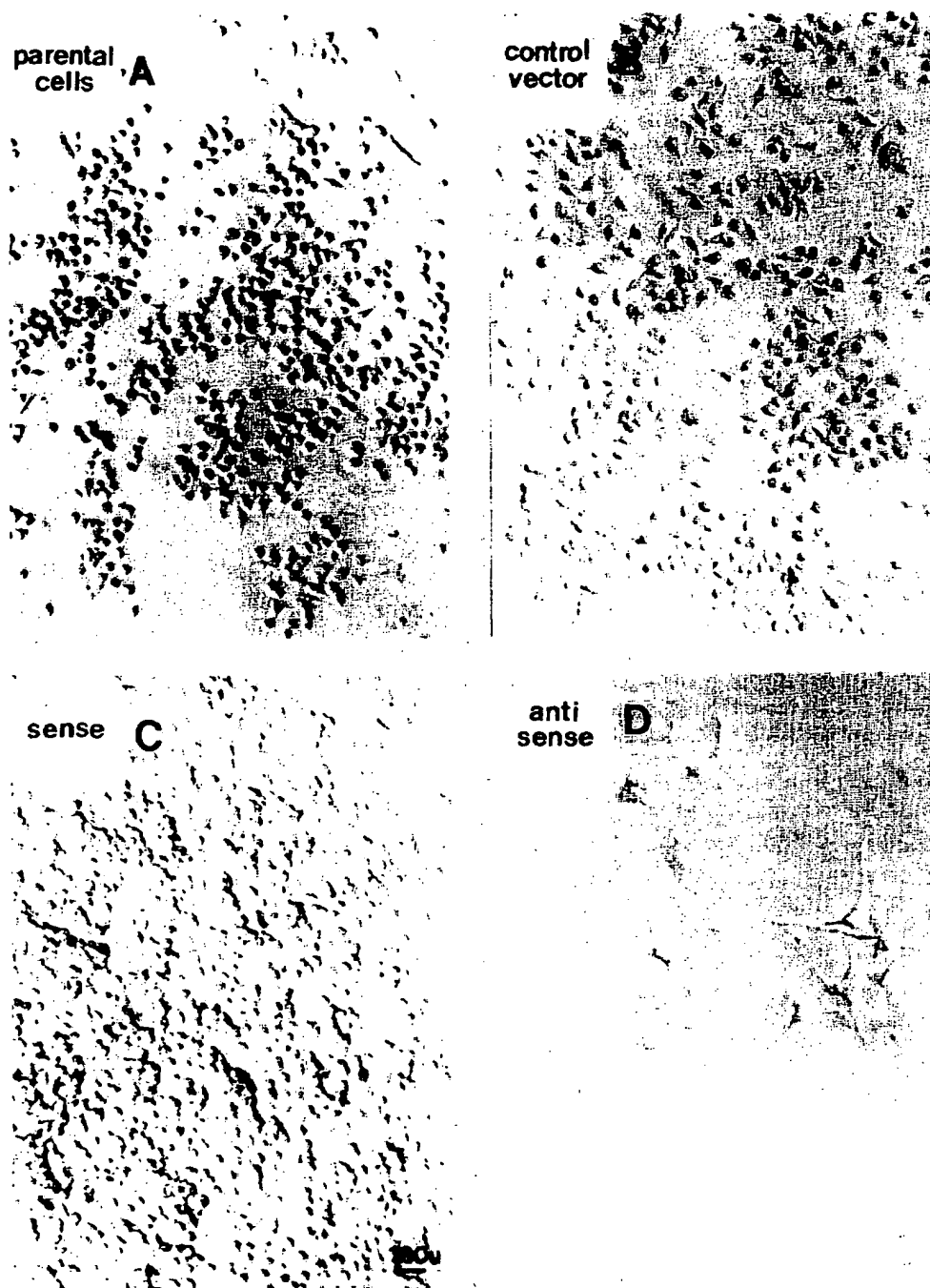
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Fig. 8. Neurite outgrowth of PC12 cells stably transfected with CaM antisense vector. PC12 cells transfected with an empty control vector or with the calmodulin gene I sense or antisense vector constructs using lipofectin were subcultured on to collagen-coated 24-well tissue culture dishes at a density of 2×10^4 cells/cm^2 and grown for 8 days. The photomicrographs show that the morphology of untransfected parental PC12 cells (A) was similar to that of cells containing control vector (B) or calmodulin sense vector (C), whereas neuritic processes were observed in the cells transfected with the calmodulin antisense vector (D). Scale bar, 100 μm (from Davidkova *et al.*, 1996).

mediated by these receptors. These results suggest that antisense oligodeoxynucleotides may also be useful as therapeutic agents to reverse disorders associated with dopaminergic hyperactivity. The encouraging biological effects obtained in in vitro studies with antisense RNA expression vectors suggest that this may be a suitable and potentially more practical strategy to block the expression of genes specifically in the nervous system in vivo.

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